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(54) Title: SPHINGOSINE KINASE, CLONING, EXPRESSION AND METHODS OF USE

(57) Abstract

The present invention relates to nucleic acids encoding sphingosine kinase (SPHK) and methods for use in producing sphingosine–1–phosphate (SPP), screening for inhibitors of SPHK, and as a therapeutic agent.

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TITLE OF THE INVENTION

Sphingosine Kinase, Cloning, Expression and Methods of Use

Field of the Invention

The present invention relates to nucleic acid molecules encoding sphingosine kinase including mutants, variants, fragments and derivatives thereof, and to vectors and host cells comprising such nucleic acid molecules; methods of using sphingosine kinase due to its effects on cell motility, cell proliferation and cell death; method for screening for inhibitors of sphingosine kinase; and kits comprising the compositions or polypeptides of the invention.

The present invention also relates to methods for making sphingosine-1-phosphate using the compositions of the present invention and kits for use in such methods.

Background of the Invention

Sphingosine-1-phosphate (SPP), a sphingolipid metabolite, which regulates diverse biological processes, such as cell growth, differentiation, survival, motility, and calcium mobilization, is now emerging as a new member of a class of lipid signaling molecules with novel dual intra and intercellular actions (Spiegel et al., 1998, *Ann. NY Acad. Sci.* 845:11-18). This phosphorylated derivative of sphingosine, the structural backbone of all sphingolipids, has many of the hallmarks of classical second messengers (reviewed in Spiegel and Milstien, 1995, *J. Membr. Biol.* 146, 225-237). The level of SPP is very low in cells and is rapidly increased by

activation of sphingosine kinase (SPHK), the enzyme responsible for the formation of SPP. SPHK is a member of a novel but highly conserved gene family and is distinct from other known lipid kinases. SPHK is induced by diverse physiological stimuli, including platelet-derived growth factor (PDGF) (Coroneos et al., 1995, *J. Biol. Chem.* 270, 23305; Olivera and Spiegel, 1993, *Nature*, 365, 557; Bornfeldt et al., 1995, *J. Cell Biol.* 130, 193; Pyne et al., 1996, *Eur. J. Biochem.* 237, 819), nerve growth factor (NGF) (Edsall et al., 1997, *J. Neurosci.* 17, 6952; Rius et al., 1997, *FEBS Lett.* 417, 173), muscarinic acetylcholine agonists (Meyer zu Heringdorf et al., 1998, *EMBO J.* 17, 2830-2837); TNF-α (Xia et al., 1998, *Proc. Natl. Acad. Sci. USA* 95, 14196), activation of protein kinase C (PKC) (Buehrer et al., 1996, *Biochim. Bophys. Acta* 1303, 233; Mazurek et al., 1994, *Biochem. Biophys. Res. Commun.* 198, 1), and cross-linking of the immunoglobulin receptors FcεR1 (Choi et al., 1996, Nature, 380, 634) and FcγR1 (Melendez et al., 1998, *J. Biol. Chem.* 273, 9393). Similar to other signaling molecules, SPP has a short half life due to rapid turnover catalyzed by SPP lyase and/or SPP phosphatase (Mandala et al., 1998, *Proc. Natl. Acad. Sci. USA* 95, 150; Saba et al., 1997, *J. Biol. Chem.* 272, 26087; Van Veldhoven and Mannaerts, 1991, *J. Biol. Chem.* 266, 12502; Van Veldhoven and Mannaerts, 1994, *Biochem. J.* 299, 597; Zhou and Saba, 1998, *Biochem. Biophys. Res. Commun.* 242, 502). Moreover, prevention of SPP formation by competitive inhibitors of SPHK, blunted the mitogenic response to PDGF (Olivera and Spiegel, 1993, supra; Rani et al., 1997, *J. Biol. Chem.* 272, 10777), the cytoprotective effects of NGF (Edsall et al., 1997, supra), vitamin D3 (Kleuser et al., 1998, *Cancer Res.* 58, 1817), PKC, and cAMP activators

(Cuvillier et al., 1996, Nature, 381, 800; Machwate et al., 1998, *Mol. Pharmacology* 54, 70), as well as calcium mobilization induced by FcεR1, FcγR1, and muscarinic acetylcholine receptors (Choi et al., 1996, supra; Melendez et al., 1998, supra; Meyer zu Heringdorf et al., 1998, supra). In addition, microinjected SPP mobilizes calcium from internal sources (Meyer zu Heringdorf et al., 1998, supra), is mitogenic for Swiss 3T3 fibroblasts (Van Brocklyn et al., 1998, *J. Cell Biol.* 142, 229), and inhibits apoptosis of mouse oocytes induced by the anti-tumor drug doxorubicin (Perez et al., 1997, *Nature Med.* 3, 1228). Collectively, these data indicate that SPP acts intracellularly to regulate calcium mobilization, cell growth, and survival.

In addition to its role as an intracellular mediator, interest in SPP has been stimulated by our discovery of the family of cell surface G-protein coupled receptors, encoded by the endothelial differentiation genes (edg), that specifically bind SPP with high affinity and specificity (Lee et al., 1998, *Science* 279, 1552; Van Brocklyn et al., 1998, *J. Cell Biol.* 142, 229; Van Brocklyn et al., 1999, *J. Biol. Chem.* 274, 4626), supporting an additional role for SPP as an extracellular mediator. Previously, some of the biological effects of SPP when added exogenously, such as inhibition of platelet activation and motility and cell shape changes (Yamamura et al., 1997, *Biochemistry* 36, 10751), induction of neurite retraction and soma rounding (Postma et al., 1996, *EMBO J.* 15, 2388; Sato et al., 1997, *Biochem. Biophys. Res. Commun.* 240, 329), and activation of G_i protein-gated inward rectifying K^+-channels in atrial myocytes (van Koppen

et al., 1996, *J. Biol. Chem.* 266, 12502), have been attributed to interactions with putative cell surface receptors. SPP receptors couple to three different Gαs and βγ dimers to signal through cAMP, Ras, and mitogen-activated protein kinase, Rho, phospholipase C, and several protein tyrosine kinases (Goetzl and An, 1998, *FASEB J.* 12, 1589; Gonda et al., 1999, *Biochem. J.* 337, 67; Lee et al., 1998, *Science* 279, 1552; Okamoto et al., 1998, *J. Biol. Chem.* 273, 27104; Van Brocklyn et al., 1998, supra; Zondag et al., 1998, *Biochem. J.* 330, 605). Furthermore, SPP is stored in high concentrations in human platelets, from which it is released upon activation by physiological stimuli (Yatomi et al., 1997a, *J. Biochem.* 86, 193; Yatomi et al., 1997b, *J. Biol. Chem.* 272, 5291), suggesting that SPP can also be considered as an autocrine factor involved in endothelial injury, inflammation, thrombosis, and angiogenesis.

Collectively, these observations provide new insights into the biological functions of SPP and emphasize the importance of SPHK, the enzyme that regulates its formation. Until recently, it had been difficult to critically evaluate the second messenger roles of SPP because most of the relevant enzymes involved in its metabolism had not been purified and cloned. Thus, much of the evidence which led to the elucidation of its importance has been indirect, relying on exogenous application and the use of inhibitors, and is further complicated by the existence of cell surface receptors. Therefore, there is a need for SPHK for a direct evaluation of SPP.

Summary of the Invention

The present invention meets the need described above. We recently purified SPHK to apparent homogeneity from rat kidney (Olivera et al., 1998, *J. Biol. Chem.* 273, 12576-12583) and subsequently cloned and characterized the first mammalian SPHKs (murine SPHK1a and SPHK1b) (Kohama et al., 1998, *J. Biol. Chem.* 273, 23722-23728). Sequence analyses identified homologs of SPHK in numerous widely disparate organisms, demonstrating that SPHK is a member of a novel but highly conserved gene family and is distinct from other known lipid kinases. Expression of SPHK caused a decrease in the levels of sphingosine and, to a lesser extent, ceramide, with a concomitant marked increase in the mass of endogenous SPP (Kohama et al., 1998, supra) and can be used to clarify its intracellular functions. Here we show that transfection of cells with a SPHK expression vector confers serum-independent growth, increases proliferation, and suppresses serum-deprivation or ceramide-induced apoptosis. Of particular interest is our discovery that intracellular formation of SPP inhibits motility and chemotaxis, events which were previously ascribed to binding of SPP to cell surface receptors.

Therefore, it is an object of the present invention to provide a DNA fragment of about 1.9 kb encoding murine sphingosine kinase SPHK1a (381 amino acids) and SPHK1b (388 amino acids) deposited in GenBank at accession number AF068748 for SPHK1a and accession number Af068749 for SPHK1b. The DNA fragment is useful as a diagnostic agent, an agent for preparation of sphingosine-1-phosphate, and a therapeutic agent.

It is another object of the invention to provide an amino acid sequence for SPHK1a and SPHK1b protein encoding 381 and 388 amino acids, respectively.

It is another object of the invention to provide a recombinant vector comprising a vector and the above described DNA fragments.

It is a further object of the present invention to provide a host cell transformed with the above-described recombinant DNA construct.

It is another object of the present invention to provide a method for producing sphingosine kinase which comprises culturing a host cell under conditions such that the above-described DNA fragment is expressed and sphingosine kinase is thereby produced, and isolating sphingosine kinase for use as a reagent, for example for screening of drugs and inhibitors of sphingosine kinase and preparation of sphingosine-1-phosphate, for diagnosis, and for therapy.

It is a further object of the present invention to provide an antibody to the above-described recombinant sphingosine kinase protein.

It is yet another object of the present invention to provide a method for detecting sphingosine kinase in a sample comprising:

(i) contacting a sample with antibodies which recognize sphingosine kinase; and

(ii) detecting the presence or absence of a complex formed between sphingosine kinase and antibodies specific therefor.

It is a further object of the present invention to provide a diagnostic kit comprising an antibody against sphingosine kinase and ancillary reagents suitable for use in detecting the presence of sphingosine kinase in cells, tissue or serum from yeast, mammals, animals, birds, fish, and plants.

It is yet another object of the present invention to provide a method for the detection of sphingosine kinase from a sample using the polymerase chain reaction.

It is a further object of the present invention to provide a diagnostic kit comprising primers or oligonucleotides specific for sphingosine kinase RNA or cDNA suitable for hybridization to sphingosine kinase RNA or cDNA and/or amplification of sphingosine kinase sequences and ancillary reagents suitable for use in detecting sphingosine kinase RNA/cDNA in mammalian tissue.

It is yet another object of the present invention to provide a method for the detection of sphingosine kinase in a sample which comprises assaying for the presence or absence of sphingosine kinase RNA or cDNA in a sample by hybridization assays.

It is an object of the present invention to provide a method for the measurement of sphingosine-1-phosphate (SPP) in a sample using the recombinant sphingosine kinase of the present invention. The method comprises separating SPP form other phospholipids, converting isolated SPP to sphingosine to produce converted SPP, rephosporylating coverted SPP using a detectably labeled phosphate, and measuring the detectable label produced.

It is still another object of the present invention to provide a method for increasing the amount of SPP in a cell by providing into the cell a recombinant SPHK.

It is yet another object of the present invention to provide a method for increasing or reducing cell death by decreasing or increasing the level of SPHK in said cell, respectively.

It is yet another object of the present invention to provide a method for increasing or decreasing cell proliferation by increasing or decreasing the level of SPHK in said cell, respectively.

It is still another object of the invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from increased cell death, said method comprising providing to an individual in need of such treatment an effective amount of a SPHK in a pharmaceutically accepatable diluent.

It is another object of the present invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from decreased cell death, said method comprising providing to an individual in need of such treatment an effective amount of an antibody against SPHK or an agent which inhibits SPHK expression or function in a pharmaceutically acceptable excipient.

It is another object of the present invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from decreased cell proliferation, said method comprising providing to an individual in need of such treatment an effective amount of a SPHK in a pharmaceutically acceptable diluent.

It is another object of the present invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from increased cell proliferation, said method comprising providing to an individual in need of such treatment an effective amount of an antibody against SPHK or an agent which inhibits SPHK expression or function in a pharmaceutically acceptable excipient.

It is still another object of the present invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from abnormal migration or motility of cells such as cancer, restenosis, or diabetic neuropathy, said method comprising providing to an individual in need or such treatment an effective amount of an antibody against SPHK or an agent which inhibits SPHK expression or function in a pharmaceutically acceptable diluent.

It is yet a further object of the present invention to provide a cDNA sequence encoding sphingosine kinase and vectors incorporating all or a fragment of said sequence, and cells, prokaryotic and eukaryotic, transformed or transfected with said vectors, for use in screening agents and drugs which inhibit expression or function of sphingosine kinase in such cells.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1. Predicted amino acid sequences of two murine sphingosine kinases (SEQ ID NO:1 and SEQ ID NO:2). The numbers on the right refer to the amino acid sequence of SPHK1a. Sequences homologous to the 8 tryptic peptides (Table 1) that were obtained from purified rat kidney sphingosine kinase are underlined. Calmodulin binding motifs are indicated by dashes. Potential PKC and cAMP-dependent phosphorylation sites are indicated by (*) and (◊), respectively.

Figure 2. Domain structure of sphingosine kinase. Alignment of the conserved subdomains of sphingosine kinases from various species. The conserved regions of sphingosine kinase (designated C1-C5) are shown as boxes. Dashes represent gaps in sequences. Sequences were aligned by the CLUSTALW: Multiple Sequence Alignment program. The putative human sphingosine kinase sequence was obtained by assembling sequences from several human ESTs (accession numbers D31133, AA232791, W63556, AA081152 and AA026479).

Figure 3. Tissue specific expression of sphingosine kinase. A sphingosine kinase probe was hybridized to poly(A)$^+$ RNA from the indicated mouse tissues (2 μg/lane) as described in Experimental Procedures. Lanes: 1, heart; 2, brain; 3, spleen; 4, lung; 5, skeletal muscle; 6, liver; 7, kidney; 8, testis. The numbers underneath indicate relative levels of expression compared to background as quantified with the Molecular Dynamics PhosphorImager.

Figure 4. Activity of sphingosine kinase expressed in HEK293 cells.
HEK293 cells were transiently transfected with the pCMVSPORT2 expression vector alone (open circles) or containing either the SPHK1a cDNA (pCMVSPORT2sphk1a, filled triangles) or the SPHK1b cDNA (pCMVSPORT2sphk1b, filled squares). At the indicated times, sphingosine kinase activity in cell lysates was measured as described under "Experimental Procedures". Data are means of duplicates and are representative of three independent experiments.

Figure 5. Distribution of sphingosine kinase in transfected HEK293 cells. Sphingosine kinase activity in cytosolic (open bars) and membrane

(filled bars) fractions. The data are expressed as percent of the total activity.

Figure 6. Sphingosine kinase has marked specificity for D-(+)-*erythro*-sphingosine. (A) HEK293 cells were transfected with pCMVSPORT2sphk1a (filled bars) or pCMVSPORT2sphk1b (open bars) and sphingosine kinase-dependent phosphorylation of various sphingosine analogs or other lipids (50 μM) was measured in cell lysates as described under "Experimental Procedures". Data are expressed as percentage of phosphorylation of D-*erythro*-sphingosine. **Competitive inhibition of recombinant sphingosine kinase by N,N-dimethylsphingosine (B) and D,L-*threo*-dihydrosphingosine (C).** Sphingosine kinase activity in HEK293 cell lysates after transfection with pCMVSPORT2sphk1a was measured with varying concentrations of D-*erythro*-sphingosine in the absence (open circles), or presence of 20 μM (filled squares) or 40 μM (filled triangles) N,N-dimethylsphingosine (B), or presence of 10 μM (filled squares) or 20 μM (filled triangles) D,L-*threo*-dihydrosphingosine (C). Data are means ± SD. Inserts are double reciprocal plots. Km (μM) and Vmax (nmol/min/mg) values: D-(+)-*erythro*- sphingosine, 2.15 and 46.7. Ki values are 2.12 for D,L-*threo*-dihydrosphingosine and 2.64 for N,N-dimethylsphingosine.

Figure 7. Changes in mass levels of sphingolipid metabolites in NIH 3T3 cells overexpressing sphingosine kinase. Mass levels of SPP **(A)**, sphingosine **(B)**, and ceramide **(C)** in NIH 3T3 cells transfected with empty vector (Control) or with pCMVSPORT2sphk1a were measured after 48 h as described under "Experimental Procedures".

Figure 8. Sphingosine kinase expression results in an increase in intracellular but not extracellular levels of sphingosine-1-phosphate. (A) Cytosolic SPHK activity was measured in cells stably transfected with c-myc-pcDNA3-SPHK1a (open bars) or c-myc-pcDNA3 (filled bars). The SPHK activity in vector transfected cells was 108 ± 12 and 111 ± 7 pmol/min/mg for NIH 3T3 and HEK293 cells, respectively. *Inset*: Western blot showing expression of SPHK. Lanes 1,2 - vector-transfected HEK293 and NIH 3T3 cells, respectively; lanes 3,4 - SPHK1a-transfected HEK293 and NIH 3T3 cells, respectively. **(B)** SPP levels in cells stably expressing SPHK. Cells were washed and incubated for 24 h in serum-free media (HEK293 cells), or serum-free media containing fatty acid free BSA (20 µg/ml), transferrin (2 µg/ml), and insulin (2 µg/ml) (NIH 3T3 fibroblasts). SPP was extracted and measured as described in Materials and Methods. Levels of SPP in vector-transfected cells were 0.119 ± 0.003, and 0.012 ± 0.002 pmol/nmol phospholipid for NIH 3T3 and HEK293 cells, respectively. **(C)** Cellular and secreted [^{32}P]SPP. Autoradiograms of TLC analyses of [^{32}P]SPP extracted from HEK293 cells labeled to isotopic equilibrium with [^{32}]P_i. Duplicate cultures were treated with 5 µM sphingosine (Sph) 10 min prior to extraction of lipids (lanes 2,4,6,8).

Figure 9. Cellular localization of c-myc-sphingosine kinase. NIH3T3 fibroblasts transiently or stably expressing SPHK and HEK293 cells stably expressing SPHK were incubated with a monoclonal c-myc antibody (20 µg/ml), and stained with anti-mouse Texas Red-conjugated IgG (1:100 dilution) (left panels) or anti-mouse FITC-monoclonal IgG (1:10 dilution) (right panels). Fluorescence micrographs

(60X) were taken using a Nikon Eclipse TE200 inverted fluorescent microscope. Vector transfectants (c-myc-pcDNA3) did not show any significant fluorescence.

Figure 10. Expression of sphingosine kinase stimulates DNA synthesis. (A) NIH 3T3 fibroblasts were transiently transfected with empty vector (pCMV-SPORT2, filled bars) or pCMV-SPORT2-SPHK1a (open bars) together with pCEFL-GFP. Cells were serum-starved for 18 h and incubated in serum-free media without (None) or with PDGF (1 ng/ml), FBS (0.1%), or SPP (10 μM). After 16 h, BrdU was added for an additional 3 h. Double immunofluorescence was used to visualize transfected cells and BrdU incorporation, and the proportion of cells incorporating BrdU among total transfected cells (expressing the green fluorescent protein GFP) was determined. Data are means ± SD of duplicate cultures from a representative experiment. At least three different fields were scored with a minimum of 100 cells scored per field. Similar results were obtained in three independent experiments. **(B)** Representative images of PDGF-treated cells. Vector- and SPHK-transfected NIH 3T3 cells expressing GFP (left panels) and incorporating BrdU (right panels) were visualized by double immunofluorescence. Arrows indicate cells which are positive for both.

Figure 11. Expression of sphingosine kinase stimulates growth of NIH 3T3 fibroblasts. (A) Cells stably transfected with c-myc-pcDNA3 or with c-myc-SPHK-pcDNA3 were plated at low density, washed after 24 h, and cultured in 0.5% calf serum for the indicated days. Media was replaced every two days and the cells were pulsed 18 h after the last media change with [^{3}H]thymidine for an additional 6 h. Data are means ± SD of three

independent determinations and are expressed as fold increase of the value determined after 1 day. Similar results were obtained in three independent experiments. **(B)** Representative images of cells stably expressing c-myc-SPHK or transfected with vector after 1 and 6 days in 0.5% calf serum. Micrographs were taken using a phase contrast microscope at 40X magnification.

Figure 12. Expression of sphingosine kinase reduces apoptosis. (A) NIH 3T3 fibroblasts were transiently transfected with vector (filled squares) or SPHK (open squares), together with pCEFL-GFP, as described in the legend to Fig. 3, and serum starved for the indicated times. Total GFP-expressing cells and GFP-expressing cells displaying fragmented nuclei indicative of apoptosis were counted (Van Brocklyn et al., 1999, *J. Biol. Chem.* 274, 4626-4632). A minimum of 500 cells in each field were scored. Data are mean ± SE of three independent experiments, each one done in duplicate or triplicate. **(B)** Mass pools of stably vector transfected (filled bars) or c-myc-tagged SPHK (open bars) transfected NIH 3T3 cells were serum starved in the absence (None) or presence of 50 nM staurosporine (Staur) or 10% serum plus 250 nM staurosporine (CS+Staur) for 24 h. Percentages of apoptotic cells were determined by Hoechst staining and visualization by fluorescence microscopy. **(C)** Note the typical condensed fragmented nuclei of apoptotic cells in vector but not in SPHK overexpressing cells after serum deprivation. **(D)** Mass pools of HEK293 cells stably expressing c-myc-tagged SPHK were incubated in serum-free medium for 30 h, then treated in the absence (None) or presence of 25 μM C2-ceramide (C2-Cer) or 100 nM staurosporine (Staur) for 24 h and

stained with Hoechst. Data are means ± SE of four independent experiments, each one done in triplicate.

Figure 13. Expression of sphingosine kinase inhibits chemotactic motility of NIH 3T3 fibroblasts. (A) NIH 3T3 fibroblasts transfected with empty vector (filled bars) or pCMV-SPORT2- SPHK1a (open bars) were trypsinized 24 h after transfection, washed with serum free media and chemotaxis towards PDGF (60 ng/ml) or 10% serum (FBS) was measured in a Boyden chamber after 24 h (PDGF) or 6 h (FBS) as described in Materials and Methods. **(B)** NIH 3T3 cells stably transfected with c-myc-pcDNA3 (filled bars) or c-myc-pcDNA3-SPHK (open bars) were trypsinized, washed with serum free media and placed in a Boyden chamber. Chemotaxis towards PDGF (60 ng/ml) or 10% serum (FBS) and chemokinesis (None) were measured after 24 h. Data are mean ± SD of duplicate determinations. Each determination is the average of three random microscope fields. Similar results were found in three independent experiments. **(C)** NIH 3T3 cells stably transfected with c-myc-pcDNA3 (filled squares) or c-myc-pcDNA3-SPHK (open squares) were detached from the dishes, washed with serum-free media and attachment of cells to collagen I coated plates was determined at the indicated times. **(D)** Attachment of NIH 3T3 cells stably transfected with c-myc-pcDNA3 (filled bars) or c-myc-pcDNA3-SPHK (open bars) to collagen I, fibronectin, polylysine, and Matrigel after 30 min was determined as described in Materials and Methods.

Figure 14. Standard curve for SPP. (A) Autoradiogram of a TLC demonstrating increased formation of [^{32}P]SPP (Rf = 0.25 ± 0.01) with increasing amounts of standard SPP added (numbers indicate pmol SPP). Arrow indicates the location of

SPP visualized with molybdenum blue spray and O indicates origin. **(B) Standard curves for analysis of SPP.** Radioactive spots corresponding to [^{32}P]SPP on the TLC shown in A were quantified with a phosphoimager. **(Insert) Lack of effects of lipids from different sources on SPP determination.** The indicated concentrations of standard SPP were added to: control alkaline extract (no cells, open circles); alkaline lipid extract from 20 x 10^6 Jurkat cells (filled squares); alkaline lipid extract from 5 x 10^6 PC12 cells (filled triangles). In another experiment, 80 nmol total lipid extract from Jurkat cells (closed diamonds) was added prior to the final enzymatic step. (C) **Quantitation of cellular SPP as a function of increasing cell numbers.** SPP levels in the indicated numbers of U937 cells were determined as described in Materials and Methods.

Figure 15. Levels of SPP in serum. (A) TLC showing [^{32}P]SPP in horse serum (HS), fetal bovine serum (FBS) or SPP standard (Std) without or with alkaline phosphatase (50 units) treatment. Arrow indicates the location of SPP visualized with molybdenum blue spray, and O indicates origin. (B) Quantitation of levels of SPP in horse serum (HS), fetal bovine serum (FBS), calf serum (CS), or charcoal stripped calf serum (CCS) were determined as described in Materials and Methods. Data are the mean ± SD of triplicate values. Similar results were obtained in three independent experiments.

Figure 16. Levels of SPP and sphingosine in rat tissues. SPP **(A)** and sphingosine **(B)** levels were measured in various rat tissues as described in Materials and Methods and are expressed as pmol/mg wet weight. Results are means ± SD of triplicate

determinations. Similar results were obtained in two different experiments. Total cellular phospholipids (nmol/mg wet weight) present in lipid extracts from brain, heart, testes, liver, spleen, kidney and eye were 872 ± 59, 231 ± 13, 268 ± 18, 323 ± 18, 260 ± 16, 455 ± 71 and 19 ± 2, respectively.

Figure 17. Measurement of changes in intracellular levels of SPP after treatment of PC12 cells with exogenous sphingosine (A) or NGF (B). PC12 cells (5 x 10^6) were treated with sphingosine (1 μM) for the indicated time periods. Lipids were extracted and SPP levels were analyzed as described in Materials and Methods. (B) PC12 cells (5 x 10^6) were stimulated without or with NGF (100 ng/ml) for the indicated times. Lipids were extracted and SPP content was determined.

DETAILED DESCRIPTION

In one embodiment, the present invention relates to a DNA or cDNA segment which encodes sphingosine kinase. The 49 kD sphingosine kinase polypeptide, purified from rat kidney, was excised from an SDS gel and subjected to trypsin digestion. The resulting peptides were separated by microcapillary reverse-phase HPLC and sequences of 8 peptides were determined by Edman degradation or MALKI mass spectrometry (TABLE 1). Homology searches (BLAST) against a comprehensive nonredundant database revealed no matches to known proteins. However, when the database of expressed sequence tags (dbEST) at NCBI was searched using the tBLASTn algorithm, an EST (GenBank accession number AA011725) containing sequences homologous to 3 of the 8 peptides (peptides 5 (SEQ ID NO:3), 2 (SEQ ID NO:4), and 4 (SEQ ID NO:5)) was retrieved. A further search with peptides 1 (SEQ ID NO:6), 3 (SEQ ID NO:7) and 7

(SEQ ID NO:8) yielded four additional ESTs (GenBank accession numbers AA000819, AA107451, AA592274 and AA389543). The nucleotide sequences of mouse ESTs AA000819 and AA592274 were then used to search dbEST to obtain EST AA389187. Clones AA107451 and AA389187 were highly homologous at their 3' ends, but were slightly divergent at their 5' ends. Sequencing of the full-length cDNAs revealed complete open reading frames coding for 381 and 388 amino acid peptides distributed throughout the protein, and these are thus designated SPHK1a (SEQ ID NO:1) and SPHK1b (SEQ ID NO:2) (Figure 1). In addition, both contained a portion of peptide 8 (SEQ ID NO:9). SPHK1a and 1b have predicted pIs of 6.68 and 6.89 and MWs of 42344 and 43254, respectively, in agreement with the MW of purified rat kidney sphingosine kinase (Olivera, et al., *J. Biol. Chem.* 273, 12576-12583, 1998). Multiple in-frame stop codons were identified in the 5' untranslated region of SPHK1a, as well as a Kozak concensus sequence, suggesting that this cDNA clone encodes full-length sphingosine kinase. Because SPHK1b only differs by a few amino acids at the N terminus, it may arise by alternative splicing.

TABLE I. PEPTIDE SEQUENCES OF RAT KIDNEY SPHINGOSINE KINASE

Peptide	Analysis	Sequence	[a]Peptide Mass	Theor. Mass
1	Edman	V L V L L N P R		
2	Edman	I Y Q G Q L A Y L P V G K		
3	Edman	L F Q S R		
4	Edman	I P A S S L A Q K		
5	Edman	F T V G T F F R		
6	MS-MS	Y I/L W F/M V S G S S D S S P G R	1643.76	1643.83
7	MS-MS	(R,V) P L/I L/I E E A E V S F K	1515.8	1516.0
8	MS-MS	(228.1 Da) A F/m Q/k (212.1 Da) C PR	1217.6	1217.5

Peptide sequences were determined by Edman sequencing or MALDI mass spectrometry. Cysteine residues were alkylated with isopropylacetamide. In peptide 8, the gaps could not be unambiguously defined, although the ion series defined the indicated molecular weights for gaps.
[2] Masses represent the average isotopic mass.

A database search identified homologs of sphingosine kinase in numerous widely disparate organisms, demonstrating that sphingosine kinase is a member of a novel but highly conserved gene family. A comparison of sequences from *Saccharomyces cerviseiae* (Bunting, M. et al., *J. Biol. Chem.* 271, 10230-10236, 1996), from *C. elegans* and *S. pombe*, reveals the location of several blocks of highly conserved amino acids, one or more of which might constitute critical portions of catalytic or substrate binding sites. Five regions, in particular C1-C5, are highly conserved in all of these sequences. C1 contains an invariant positively charged motif, GGKGK, which may be part of the ATP binding site.

Thus, one aspect of the invention provides an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence selected from (a) a nucleotide sequence encoding a full length SPHK1a polypeptide having the complete amino acid sequence in Figure 1, or the complete amino acid sequence encoded by the cloned DNA in GenBank accession no. AF068748 (SEQ ID NO:10); and (b) a nucleotide sequence encoding a full length SPHK1b nucleotide sequence having the complete amino acid sequence in Figure 1, or the complete amino acid sequence encoded by the cloned DNA in GenBank accession no. Af068749 (SEQ ID NO:11).

In addition, isolated nucleic acid molecules of the invention include DNA molecules which comprise a

sequence substantilly differenct from those described above but which, due to the degeneracy of the genetic code, still encode sphingosine kinase proteins. Of course, the genetic code and species-specific codon preferences are well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate variants described above, for instance, to optimize codon expression for a particular host (e.g., change codons in the human mRNA to those preferred by a bacterial host such as *E.coli*).

Nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the antisense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

The present invention is further directed to nucleic acid molecules encoding portions or fragments

of the nucleotide sequences described herein. Fragments include portions of the nucleotide sequences of Figure 1 at least 10 contiguous nucleotides in length selected from any two integers, one of which representing a 5' nucleotide position and a second of which representing a 3' nucleotide position, where the first nucleotide for each nucleotide sequence in Figure 1 is position 1. That is, every combination of a 5' and 3' nucleotide position that a fragment at least 10 contiguous nucleotide bases in length or any interger between 10 and the length of an entire nucleotide sequence of SPHK1a or SPHK1b of Figure 1 minus 1.

Further, the invention includes polynucleotides comprising fragments specified by size, in nucleotides, rather than by nucleotide positions. The invention includes any fragment size, in contiguous nucleotides, selected from intergers between 1- and the entire length of an entire nucleotide sequence minus 1. Preferred sizes include 20-50 nucleotides, 50-300 nucleotides useful as primers and probes. Regions from which typical sequences may be derived include but are not limited to, for example, regions encoding specific epitopes or domains within said sequence, such as domains C1-C5 shown in Figure 2.

In another aspect, the invention provides isolated nucleic acid molecules comprising polynucleotides which hybridize under stringent hybridization conditions to a polynucleotide sequence of the present invention described above, for instance, a nucleic acid sequences shown in Figure 1 or a specified fragment thereof. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5X SSC (150 mM NaCl, 15 mM trisodium

citrate), 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured sheared salmon sperm DNA, followed by washing the filters in 0.1X SSC at about 65°C.

The sequences encoding the polypeptides of the present invention or portions thereof may be fused to other sequences which provide additional functions known in the art such as a marker sequence, or a sequence encoding a peptide which facilitates purification of the fused polypeptide, peptides having antigenic determinants known to provide helper T-cell stimulation, peptides encoding sites for post-tranlational modifications, or amino acid sequences which target the fusion protein to a desired location, e.g. a heterologous leader sequence.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the sphingosine kinase polypeptides shown in Figure 1. Variant may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus of a chromosome of an organism. Non-naturally occuring variants may be produced by known mutagenesis techniques. Such variants include those produced by nucleotide substitution, deletion, or addition of one or more nucleotides in the coding or noncoding regions or both. Alterations in the coding regions may produce conservative or nonconservative amino acid substitutions, deletions, or additions. Especially preferred among these are silent substitutions, additions, and deletions which do not alter the properties and activities of sphingosine kinase polypeptides disclosed herein or portions thereof.

Also preferred in this regard are conservative substitutions.

Nucleic acid molecules with at least 90-99% identity to a nucleic acid shown in Figure 1 is another aspect of the present invention. These nucleic acids are included irrespective of whether they encode a polypeptide having sphingosine kinase activity. By "a polypeptide having sphingosine kinase activity" is intended polypeptides exhibiting activity similar, but not identical, to an activity of the sphingosine kinase of the invention, as measured in the assays described below. The biological acitivity or function of the polypeptides of the present invention are expected to be similar or identical to polypeptides from other organisms that share a high degree of structural identity/similarity.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The vector can take the form of a plasmid, phage, cosmid, YAC, eukaryotic expression vector such as a DNA vector, *Pichia pastoris*, or a virus vector such as for example, baculovirus vectors, retroviral vectors or adenoviral vectors, and others known in the art. The cloned gene may optionally be placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences, or sequences which may be inducible and/or cell type-specific. Suitable promoters will be known to a person with ordinary skill in the art. The expression construct will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. Among the vectors preferred for use include pCMV-

SPORT2 (Life Technologies, Inc.), pcDNA3 (Invitrogen), to name a few.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, electroporation, infection, and other methods known in the art and described in standard laboratory manuals such as Current Protocols in Molecular Biology, Ausubel, F. M. *et al.* (Eds), Wiley & Sons, Inc. All documents cited herein supra and infra are hereby incorporated in their entirety by referece thereto.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for example, bacterial), lower eukaryotic (for example, yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to rat and human). Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences which are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning,

Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of SPHK, such as glutathione S-transferase, or a series of histidine residues also known as a histidine tag. The recombinant molecule can be suitable for transfecting eukaryotic cells, for example, mammalian cells and yeast cells in culture systems. *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as HEK293 cells , and NIH 3T3 cells, to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus (CMV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein described below.

In another embodiment, the present invention relates to a SPHK1a protein having an amino acid sequence corresponding to GenBank accession no.

AF068748 and encompassing 381 amino acids or any allelic variation thereof and SPHK1b protein having an amino acid sequence corresponding to GenBank accession no. AF068749 and encompassing 388 amino acids.

A polypeptide or amino acid sequence derived from the amino acid sequences mentioned above, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system. In addition the polypeptide can be fused to other proteins or polypeptides which increase its antigenicity, such as adjuvants for example.

As noted above, the methods of the present invention are suitable for production of any polypeptide of any length, via insertion of the above-described nucleic acid molecules or vectors into a host cell and expression of the nucleotide sequence encoding the polypeptide of interest by the host cell. Introduction of the nucleic acid molecules or vectors into a host cell to produce a transformed host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as

Davis et al., Basic Methods In Molecular Biology (1986). Once transformed host cells have been obtained, the cells may be cultivated under any physiologically compatible conditions of pH and temperature, in any suitable nutrient medium containing assimilable sources of carbon, nitrogen and essential minerals that support host cell growth. Recombinant polypeptide-producing cultivation conditions will vary according to the type of vector used to transform the host cells. For example, certain expression vectors comprise regulatory regions which require cell growth at certain temperatures, or addition of certain chemicals or inducing agents to the cell growth medium, to initiate the gene expression resulting in the production of the recombinant polypeptide. Thus, the term "recombinant polypeptide-producing conditions," as used herein, is not meant to be limited to any one set of cultivation conditions. Appropriate culture media and conditions for the above-described host cells and vectors are well-known in the art. Following its production in the host cells, the polypeptide of interest may be isolated by several techniques. To liberate the polypeptide of interest from the host cells, the cells are lysed or ruptured. This lysis may be accomplished by contacting the cells with a hypotonic solution, by treatment with a cell wall-disrupting enzyme such as lysozyme, by sonication, by treatment with high pressure, or by a combination of the above methods. Other methods of bacterial cell disruption and lysis that are known to one of ordinary skill may also be used.

Following disruption, the polypeptide may be separated from the cellular debris by any technique suitable for separation of particles in complex

mixtures. The polypeptide may then be purified by well known isolation techniques. Suitable techniques for purification include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, electrophoresis, immunoadsorption, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, immunoaffinity chromatography, size exclusion chromatography, liquid chromatography (LC), high performance LC (HPLC), fast performance LC (FPLC), hydroxylapatite chromatography and lectin chromatography.

The recombinant or fusion protein can be used as a diagnostic tool and in a method for producing sphingosine-1-phosphate, detectably labeled and unlabeled, and in a method for measuring levels of SPP in samples as described below. In addition, the recombinant protein can be used as a therapeutic agent to reduce cell death and/or increase cell proliferation. The transformed host cells can be used to analyze the effectiveness of drugs and agents which inhibit SPHK function, such as host proteins or chemically derived agents or other proteins which may interact with the cell to down-regulate or alter the expression of SPHK, or its cofactors.

In another embodiment, the present invention relates to monoclonal or polyclonal antibodies specific for the above-described recombinant proteins (or polypeptides). For instance, an antibody can be raised against a peptide described above, or against a portion thereof of at least 10 amino acids, perferrably, 11-15 amino acids. Persons with ordinary skill in the art using standard methodology can raise monoclonal and polyclonal antibodies to the protein

(or polypeptide) of the present invention, or a unique portion thereof. Material and methods for producing antibodies are well known in the art (see for example Goding, in, Monoclonal Antibodies: Principles and Practice, Chapter 4, 1986).

The level of expression of SPHK, can be detected at several levels. Using standard methodology well known in the art, assays for the detection and quantitation of SPHK RNA can be designed, and include northern hybridization assays, *in situ* hybridization assays, and PCR assays, among others. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985), or Current Protocols in Molecular Biology, Ausubel, F. M. *et al.* (Eds), Wiley & Sons, Inc. for general description of methods for nucleic acid hybridization. Polynucleotide probes for the detection of SPHK RNA can be designed from the sequence available at accession numbers AF068748 and/or AF068749 for the mouse sequence [Olivera, A., et al. *J. Biol. Chem.* 273:12576-12583]. For example, RNA isolated from samples can be coated onto a surface such as a nitrocellulose membrane and prepared for northern hybridization. In the case of *in situ* hybridization of biopsy samples for example, the tissue sample can be prepared for hybridization by standard methods known in the art and hybridized with polynucleotide sequences which specifically recognize SPHK RNA. The presence of a hybrid formed between the sample RNA and the polynucleotide can be detected by any method known in the art such as radiochemistry, or immunochemistry, to name a few.

One of skill in the art may find it desirable to prepare probes that are fairly long and/or encompass regions of the amino acid sequence which would have a high degree of redundancy in the corresponding nucleic acid sequences. In other cases, it may be desirable to use two sets of probes simultaneously, each to a different region of the gene. While the exact length of any probe employed is not critical, typical probe sequences are no greater than 500 nucleotides, even more typically they are no greater than 250 nucleotides; they may be no greater than 100 nucleotides, and also may be no greater than 75 nucleotides in length. Longer probe sequences may be necessary to encompass unique polynucleotide regions with differences sufficient to allow related target sequences to be distinguished. For this reason, probes are preferably from about 10 to about 100 nucleotides in length and more preferably from about 20 to about 50 nucleotides.

The DNA sequence of SPHK can be used to design primers for use in the detection of SPHK using the polymerase chain reaction (PCR) or reverse transciption PCR (RT-PCR). The primers can specifically bind to the SPHK cDNA produced by reverse transcription of SPHK RNA, for the purpose of detecting the presence, absence, or quantifying the amount of SPHK by comparison to a standard. The primers can be any length ranging from 7-40 nucleotides, preferably 10-15 nucleotides, most preferably 18-25 nucleotides homologous or complementary to a region of the SPHK sequence. Reagents and controls necessary for PCR or RT-PCR reactions are well known in the art. The amplified products can then be analyzed for the presence or absence of SPHK sequences, for example by gel

fractionation, by radiochemistry, and immunochemical techniques. This method is advantageous since it requires a small number of cells. Once SPHK is detected, a determination whether the cell is overexpressing or underexpressin SPHK can be made by comparison to the results obtained from a normal cell using the same method. Increased SPHK RNA levels correlate with increased cell proliferation and reduced cell death.

In another embodiment, the present invention relates to a diagnostic kit for the detection of SPHK RNA in cells, said kit comprising a package unit having one or more containers of SPHK oligonucleotide primers for detection of SPHK by PCR or RT-PCR or SPHK polynucleotides for the detection of SPHK RNA in cells by in situ hybridization or northern analysis, and in some kits including containers of various reagents used for the method desired. The kit may also contain one or more of the following items: polymerization enzymes, buffers, instructions, controls, detection labels. Kits may include containers of reagents mixed together in suitable proportions for performing the methods in accordance with the invention. Reagent containers preferably contain reagents in unit quantities that obviate measuring steps when performing the subject methods.

In a further embodiment, the present invention provides a method for identifying and quantifying the level of SPHK present in a particular biological sample. Any of a variety of methods which are capable of identifying (or quantifying) the level of SPHK in a sample can be used for this purpose.

Diagnostic assays to detect SPHK may comprise a biopsy or in situ assay of cells from an organ or tissue sections, as well as an aspirate of cells from

a tumour or normal tissue. In addition, assays may be conducted upon cellular extracts from organs, tissues, cells, urine, or serum or blood or any other body fluid or extract.

When assaying a biopsy, the assay will comprise, contacting the sample to be assayed with a SPHK ligand, natural or synthetic, or an antibody, polyclonal or monoclonal, which recognizes SPHK, or antiserum capable of detecting SPHK, and detecting the complex formed between SPHK present in the sample and the SPHK ligand or antibody added.

SPHK ligands or substrates include for example, sphingosine, in addition to natural and synthetic classes of ligands and their derivatives which can be derived from natural sources such as animal or plant extracts. Other SPHK ligands include calmodulin.

SPHK ligands or anti-SPHK antibodies, or fragments of ligand and antibodies capable of detecting SPHK may be labeled using any of a variety of labels and methods of labeling for use in diagnosis and prognosis of disease associated with increased cell proliferation, such as cancer, or reduced cell death. Examples of types of labels which can be used in the present invention include, but are not limited to, enzyme labels, radioisotopic labels, non-radioactive isotopic labels, and chemiluminescent labels.

Examples of suitable enzyme labels include malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast-alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate isomerase, peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate

dehydrogenase, glucoamylase, acetylcholine esterase, etc.

Examples of suitable radioisotopic labels include ^{3}H, ^{111}In, ^{125}I, ^{32}P, ^{35}S, ^{14}C, ^{57}To, ^{58}Co, ^{59}Fe, ^{75}Se, ^{152}Eu, ^{90}Y, ^{67}Cu, ^{21}Ci, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, ^{11}C, ^{19}F, ^{123}I, etc.

Examples of suitable non-radioactive isotopic labels include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Tr, ^{46}Fe, etc.

Examples of suitable fluorescent labels include a ^{152}Eu label, a fluorescein label, an isothiocyanate label, a rhodamine label, a phycoerythrin label, a phycodyanin label, an allophycocyanin label, a fluorescamine label, etc.

Examples of chemiluminescent labels include a luminal label, an isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, a luciferin label, a luciferase label,etc.

Those of ordinary skill in the art will know of other suitable labels which may be employed in accordance with the present invention. The binding of these labels to ligands and to antibodies or fragments thereof can be accomplished using standard techniques commonly known to those of ordinary skill in the art. Typical techniques are described by Kennedy, J. H., *et al.*,1976 (*Clin. Chim. Acta* **70**:1-31), and Schurs, A. H. W. M., *et al.* 1977 (*Clin. Chim Acta* **81**:1-40). Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleimide method, and others, all of which are incorporated by reference herein.

The detection of the antibodies (or fragments of antibodies) of the present invention can be improved through the use of carriers. Well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and

modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to SPHK. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Those skilled in the art will note many other suitable carriers for binding monoclonal antibody, or will be able to ascertain the same by use of routine experimentation.

The ligands or antibodies, or fragments of antibodies or ligands of SPHK discussed above may be used to quantitatively or qualitatively detect the presence of SPHK. Such detection may be accomplished using any of a variety of immunoassays known to persons of ordinary skill in the art such as radioimmunoassays, immunometic assays, etc. Using standard methodology well known in the art, a diagnostic assay can be constucted by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g. nitrocelluolose membrane), antibodies specific for SPHK or a portion of SPHK, and contacting it with a sample from a person suspected of having a SPHK related disease. The presence of a resulting complex formed between SPHK in the sample and antibodies specific therefor can be detected by any of the known detection methods common in the art such as fluorescent antibody spectroscopy or colorimetry. A good description of a radioimmune assay may be found in Laboratory Techniques and Biochemistry in Molecular

Biology. by Work, T.S., *et al.* North Holland Publishing Company, N.Y. (1978), incorporated by reference herein. Sandwich assays are described by Wide at pages 199-206 of Radioimmune Assay Method, edited by Kirkham and Hunter, E. & S. Livingstone, Edinburgh, 1970.

The diagnostic methods of this invention are predictive of proliferation and metastatic potential in patients suffering from cancers including carcinomas of the lung like small cell carcinoma, large cell carcinoma, squamous carcinoma, and adenocarcinoma, stomach carcinoma, prostatic adenocarcinoma, ovarian carcinoma such as serous cystadenocarcinoma and mucinous cytadenocarcinoma, ovarian germ cell tumors, testicular carcinomas, and germ cell tumors, pancreatic adenocarcinoma, biliary adenocarcinoma, heptacellular carcinoma, renal cell adenocarcinoma, endometrial carcinoma including adenocarcinomas and mixed Mullerian tumors (carcinosarcomas), carcinomas of the endocervix, ectocervix, and vagina such as adenocarcinoma and squamous carcinoma, basal cell carcinoma, melanoma, and skin appendage tumors, esophageal carcinoma, carcinomas of the nasopharyns and oropharynx including squamous carcinoma and adenocarcinomas, salivary gland carcinomas, brain and central nervous system tumors including tumors of glial, neuronal, and meningeal origin, tumors of peripheral nerve, soft tissue sarcomas and sarcoms of bone and cartilage. Cells of these tumors which express increased levels of SPHK RNA or SPHK protein, have increased proliferation and decreased cell death.

The protein can be used to identify inhibitors of SPHK activity. Using an enzyme assay as described

below in the Examples, natural and synthetic agents and drugs can be discovered which result in a reduction or elimination of SPHK enzymatic activity. Knowledge of the mechanism of action of the inhibitor is not necessary as long as a decrease in the activity of SPHK is detected. Inhibitors may include agents or drugs which either bind or sequester the enzyme's substrate(s) or cofactor(s), or inhibit the enzyme itself, directly, for example by irreversible binding of the agent or drug to the enzyme, or indirectly, for example by introducing an agent which binds the SPHK substrate. Agents or drugs related to this invention may result in partial or complete inhibition of SPHK activity. Inhibitors of SPHK include DL-*threo*-Dihydrosphingosine (DHS) and the more recently discovered inhibitor *N,N*-dimethylsphingosine (DMS) described in Edsall, L. C. et al., 1998, *Biochemistry* 37, 12892-12898. Inhibitors of SPHK may be used in the treatment or amelioration of diseases such as cancer, artherosclerosis, neurodegenerative disorders, i.e. stroke, Alzheimer's.

Agents which decrease the level of SPHK (i.e. in a human or an animal) or reduce or inhibit SPHK activity may be used in the therapy of any disease associated with the elevated levels of SPHK or diseases associated with increased cell proliferation such as cancer. An increase in the level of SPHK is determined when the level of SPHK in a tumor cell is about 2-3 times the level of SPHK in the normal cell, up to about 10-100 times the amount of SPHK in a normal cell. Agents which decrease SPHK RNA include, but are not limited to, one or more ribozymes capable of digesting SPHK RNA, or antisense oligonucleotides capable of hybridizing to SPHK RNA such that the translation of SPHK is inhibited or reduced resulting

in a decrease in the level of SPHK. These antisense oligonucleotides can be administered as DNA, as DNA entrapped in proteoliposomes containing viral envelope receptor proteins (Kanoda, Y. *et al.*, 1989, *Science* 243, 375) or as part of a vector which can be expressed in the target cell such that the antisence DNA or RNA is made. Vectors which are expressed in particular cell types are known in the art, for example, for the mammary gland, please see Furth, (1997) (*J. Mammary Gland Biol. Neopl.* 2, 373) for examples of conditional control of gene expression in the mammary gland. Alternatively, the DNA can be injected along with a carrier. A carrier can be a protein such as a cytokine, for example interleukin 2, or polylysine-glycoprotein carrier. Such carrier proteins and vectors and methods of using same are known in the art. In addition, the DNA could be coated onto tiny gold beads and said beads introduced into the skin with, for example, a gene gun (Ulmer, J. B. *et al.*, 1993, *Science* 259, 1745).

Alternatively, antibodies, or compounds capable of reducing or inhibiting SPHK, that is reducing or inhibiting either the expression, production or activity of SPHK, such as antagonists, can be provided as an isolated and substantially purified protein, or as part of an expression vector capable of being expressed in the target cell such that the SPHK-reducing or inhibiting agent is produced. In addition, co-factors such as various ions, i.e. $Ca2^{+}$ or factors which affect the stability of the enzyme can be administered to modulate the expression and function of SPHK. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, oral, rectal, or

parenteral (e.g. intravenous, subcutaneous, or intramuscular) route. In addition, SPHK-inhibiting compounds may be incorporated into biodegradable polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the SPHK-inhibiting compound is slowly released systemically. The biodegradable polymers and their use are described, for example, in detail in Brem *et al.*(1991) *J. Neurosurg.* 74, 441-446. These compounds are intended to be provided to recipient subjects in an amount sufficient to effect the inhibition of SPHK. Similarly, agents which are capable of negatively affecting the expression, production, stability or function of SPHK, are intended to be provided to recipient subjects in an amount sufficient to effect the inhibition of SPHK. An amount is said to be sufficient to "effect" the inhibition or induction of SPHK if the dosage, route of administration, etc. of the agent are sufficient to influence such a response.

In line with the function of SPHK in cell proliferation, agents which stimulate the level of SPHK, such as agonists of SPHK, may be used in the therapy of any disease associated with a decrease of SPHK, or a decrease in cell proliferation, wherein SPHK is capable of increasing such proliferation, e.g. developmental retardation.

In providing a patient with agents which modulate the expression or function of SPHK to a recipient patient, the dosage of administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of agent which is in the range of from about 1 pg/kg to

10 mg/kg (body weight of patient), although a lower or higher dosage may be administered.

A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient patient. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences [16th ed., Osol, A. ed., Mack Easton PA. (1980)]. In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the above-described compounds together with a suitable amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved through the use of polymers to complex or absorb the compounds. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate) and the concentration of macromolecules as well as the method

of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate the compounds of the present invention into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly(methylmethacrylate)microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (1980).

The present invention also provides kits for use in the diagnostic or therapeutic methods described above. Kits according to this aspect of the invention may comprise one or more containers, such as vials, tubes, ampules, bottles and the like, which may comprise one or more of the compositions of the invention.

The kits of the invention may comprise one or more of the following components, one or more compounds or compositions of the invention, and one or more excipient, diluent, or adjuvant.

Measuring SPP

Based on the increasingly diverse roles of SPP in cellular processes, methods to quantitate changes in endogenous and extracellular levels of SPP have been

instrumental in correlating its effects on physiological events. SPP has previously been quantified by mass spectrometry after phenylisothiocyanate derivatization (Van Veldhoven, P. P. et al., 1994, *FEBS Lett.* 350, 91-95) and by a liquid chromatography/ion spray ionization tandem mass spectrometric method (Mano, N. et al., 1997, *Anal. Biochem.* 244, 291-300), methods which provide sensitive analyses in the fmol range and also allow quantitation of multiple sphingolipid metabolites simultaneously. However, these highly specialized methods require instrumentation that is not readily available. Alternatively, SPP has been measured after conversion to N-[^{3}H]acetylated C2-ceramide 1-phosphate with [^{3}H]acetic anhydride (Yatomi, T. et al., 1995, *Anal. Biochem.* 230, 315-320). While this method does not require expensive technology, it lacks specificity and has lower sensitivity. Here we describe a sensitive and specific enzymatic method which can be used to measure SPP levels in various biological samples.

The sphingosine kinase described above is highly specific for the naturally occuring D-(+)-*erythro*-trans-isomer of sphingosine (Kohama, T. et al., 1998, *J. Bio. Chem.* 273, 23722-23728). D-(+)-*erythro*-dihydrosphingosine is also phosphorylated, ableit to a lesser extent, whereas other related sphingolipids are not substrates (Kohama, et al., 1998, supra). Use of the recombinant SPHK described above provides an enzymatic method which is more specific than other procedures (Van Veldhoven et al., 1994, supra; Yatomi, et al., 1995, supra) which non-selectively modify the amino group of this sphingoid base.

Therefore, in another embodiment of the present invention is provided a method for the measurement of

SPP in a sample comprising (a) partitioning the sphingosine from the sphingosine kinase in the sample. This can be achieved by extracting lipids from said sample such that SPP is in the aqueous phase, or any method of separation including HPLC, column chromatography and others known to people in the art. (b) removing a phosphate group from the SPP to produce a dephosphorylated SPP; (c) incubating the dephosphorylated SPP with recombinant sphingosine kinase such that a phosphate with a detectabe label is added to the dephosphorylated SPP; and (d) measuring said detectable label.

The method can be applied to any sample, tissue, serum or cells. For tissues, it is preferable if the tissue is homogenized prior to lipid extraction. For aqueous samples, the solution should be adjusted to a workable volume by addition of a salt,such as 1 M NaCl. such that the organic and inorganic phases are separated. The lipids can be extracted by any method as long as the SPP is in a separate phase from sphingosine. For example, a solution of chloroform/methanol/3N NaOH at a 1:1:0.1, v/v has been used in the examples below. Other methods and solutions will be known to people in the art and can be used to separate SPP from sphingosine.

Once the lipids are extracted, the phase containing the SPP is retained, and incubated under conditions which allow the removal of the phosphate group to produce a dephosphorylated SPP. A phosphatase can be used to remove the phosphate group, for example, alkaline phosphatase. Other methods from removing the phosphate group can be applied, such as chemical hydrolysis, use of a nonspecific phosphatase, and other methods known to people in the art.

After removal of the phosphate group, the dephosphorylated SPP is subjected to rephosphorylation in the presence of recombinat SPHK of the present invention and a detectably labeled phosphate source such that the phosphate added to the SPP is detectable. For example, [γ-^{32}P]ATP, or [γ-^{33}P]ATP, to name a few. Any recombinant SPHK can be used, SPHK1a, SPHK1b, or any portion or variant of these proteins retaining kinase function, as well as any sphingosine kinase from other sources, mammalian, eukaryotic, or prokaryotic, which is capable of selectively adding a phosphate group to sphingosine.

The level of SPP is determined by measuring the detectable label using methods known in the art depending on which label is utilized. Methods for measurment of label can be by a phosphorimaging, scintillation counting, colorimetric measurements, chemiluminescence, and immunostaining, to name a few.

A concentration range from 25.0 fmol to 250 umol of SPP can be detected using the method of the present invention.

It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

The following Materials and Methods were used in the Examples desribed below.

Materials - SPP, sphingosine, and N,N-dimethylsphingosine were from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). All other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Poly L-lysine and collagen were from Boehringer Mannheim. (Indianapolis, IN). Alkaline phosphatase from bovine intestinal mucosa, Type VII-NT, was from Sigma (St. Louis, MO). Serum, medium, and G418 were obtained from Biofluids, Inc. (Rockville, MD). Restriction enzymes were from New England Biolabs (Beverly, MA). Poly(A)$^+$ RNA blots of multiple mouse adult tissues were purchased from Clontech (Palo Alto, CA). Lipofectamine PLUS™ and Lipofectamine were from Life Technologies, Inc. (Gaithersburg, MD). Monoclonal antibodies against c-myc were from Zymed (San Francisco, CA), and anti-mouse Texas Red dye-conjugated goat antibody was from Jackson Immunoresearch (West Grove, PA). The Anti-Fade kit was from Molecular Probes (Eugene, OR). The bromodeoxyuridine incorporation detection kit and anti-mouse FITC-conjugated IgG were obtained from Boehringer Mannheim (Indianapolis, IN). Bisbenzimide hydrochloride (Hoechst #33258) was from Calbiochem (San Diego. CA). Silica Gel 60 G plates were from EM Sciences (Cherry Hill, NJ). Mouse 2.5S nerve growth factor was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Sprague Dawley rat tissues were from Pelfreeze (Rogers, AR).

Protein sequencing of sphingosine kinase - Purified sphingosine kinase was electrophoresed on SDS-PAGE and the Coomassie-stained 49 kd band excised. After S-carboxyamidomethylation, this band was subjected to in-gel tryptic digestion as described

(Rani, C.S. et al., 1997, *J. Biol. Chem.* 272, 10777-10783). The resulting peptide mixture was separated by microbore high performance liquid chromatography on a Zorbax C18 1.0 mm by 150-mm reverse-phase column in a Hewlett-Packard 1090 HPLC with a1040 diode array detector. Fractions were selected for sequencing based on differential UV absorbance at 205, 277, and 292 nm, and the peptide sequences were determined by automated Edman degradation (Van Brocklyn, J. R. et al, 1998, *J. Cell Biol.* 142, 229-240). Complementary peptide sequence information was also obtained on 10% of the digest mixture by collisionally induced dissociation using microcapillary HPLC electrospray ionization/tandem mass spectrometry on a Finnigan TSQ7000 triple quadrupole mass spectrometer (Rani et al, 1997, supra). Sequences of smaller peaks were determined on an Applied Biosystems Procise cLC 494 sequencer or by microcapillary HPLC-MS as above or on a Finnigan LaserMat 2000 Time-of-Flight Mass Spectrometer Matrix-assisted Laser Desorption TOF/MS (MALDI-TOFMS) (Yatomi, Y. et al., 1997, *J. Biol. Chem.* 272, 5291-5297).

Cell culture

MCF-7 human breast cancer cells were cultured as recommended by the American Type Culture Collection (ATCC). Human monoblastic leukemia U937, Jurkat, and HL60 cells were obtained from ATCC (Rockville, MD) and grown in RPMI 1640 containing 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere and routinely subcultured every 2-3 days. PC12 cells were a generous gift from Dr. Gordon Guroff (NICHD, NIH, Bethesda, MD) and were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% FBS. Human embryonic kidney cells (HEK293, ATCC CRL-1573) were cultured in DMEM supplemented with 2 mM

glutamine containing 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. NIH 3T3 fibroblasts (ATCC CRL-1658) and Swiss 3T3 cells (ATCC CCL-92) were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine supplemented with 10% calf serum (Kohama et al., 1998, supra).

Transient expression of sphingosine kinase Transfections of various cell types were carried out using Lipofectamine PLUS reagent, essentially as described by the manufacturer. For human embryonic kidney 293 cells, cells were seeded at 6 x 10^5 per well in polylysine coated 6 cluster wells. After 24 h, cells were transfected with 1 µg of vector (pCMV-SPORT2) (Life Technologies, Inc.) alone or with vectors containing sphingosine kinase constructs (pCMV-SPORT2sphk1a or pCMV-SPORT2sphk1b), and 6 µl of Lipofectamine PLUS reagent plus 4 µl of Lipofectamine reagent per well. For Swiss 3T3 and NIH 3T3 fibroblasts, cells were seeded at 3 x 10^5 per well in collagen-coated 6 well clusters. After 24 h, cells were transfected with a mixture of Lipofectamine PLUS (8 µl) and lipofectamine (8 µl) and vector alone (2 µg) or sphingosine kinase constructs (2 µg). In some cases, as a measure of transfection efficiency, cells were co-transfected with 0.5 µg of pCEFLGFP (a kind gift of Dr. Silvio Gutkind), which encodes the green fluorescent protein, and were visualized with a fluorescence microscope.

Assay of sphingosine kinase activity - 1-3 days after transfection, cells were harvested and lysed by freeze-thawing in buffer A (20 mM Tris (pH 7.4), 20% glycerol, 1 mM ß-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM ß-glycero-phosphate, 15 mM NaF, 10 µg/ml leupeptin, aprotinin and soybean

trypsin inhibitor, 1 mM PMSF, and 0.5 mM 4-deoxypyridoxine). In some experiments, cell lysates were fractionated into cytosol and membrane fractions by centrifugation at 100,000 x g for 60 min (4°C). Sphingosine kinase activity was determined in the presence of 50 μM sphingosine, 0.25% Triton X-100, and [^{32}P]ATP (10 μCi, 1 mM) containing $MgCl_2$ (10 mM) in buffer A as previously described (Meye zu Heringdorf, D. et al., 1998, *EMBO J.* 17, 2830-2837). The labeled SPP was separated by TLC on silica gel G60 with 1-butanol/ethanol/acetic acid/water (80:20:10:20, v/v) and visualized by autoradiography. The radioactive spots corresponding to authentic SPP were identified as described (Yamamura, S. et al., 1997, *Biochemistry* 36, 10751-10759) and quantified with a Molecular Dynamics Storm Phosphoimager (Sunnyvale, CA). Sphingosine kinase specific activity was expressed as pmol of SPP formed per min per mg of protein.

Lipid extractions-Cells were washed with PBS and scraped in 1 ml of methanol containing 2.5 μl conc. HCl. Lipids were extracted by adding 2 ml chloroform/1M NaCl (1:1) and 100 μl 3N NaOH or lipids were extracted with 5 ml of chloroform/methanol/1 M NaCl (2:1:2, v/v) and 40 μl conc. NH_4OH and phases separated. Ceramide, sphingosine and phospholipid levels were measured in the organic layer. The basic aqueous phase containing SPP, devoid of the majority of phospholipids and sphingosine, was transferred to a siliconized glass tube. The organic phases were re-extracted with 1 ml methanol/1M NaCl (1:1) plus 50 μl 3N NaOH, and the aqueous fractions combined. Extractions of lipids from various serum samples were performed in a similar fashion. To 0.5 ml of serum, 0.5 ml of 1M NaCl was added and lipids extracted as described above. Tissue samples were homogenized in

25 mM HCl/1 M NaCl, and aliquots were taken for protein analysis and SPP extraction.

Measurement of sphingosine- Sphingosine was measured by the sphingosine kinase method as previously described (van Koppen, C. J. et al., 1996, *J. Biol. Chem.* 271, 2082-2087). Briefly, aliquots of the organic phase containing 5-10 nmol of total phospholipids were dried under nitrogen and then resuspended in 0.25% Triton X-100. Reactions were started by addition of of [γ^{32}P]ATP (10 μCi, 10 mM) containing 10 mM $MgCl_2$, 30,000 U of partially purified rat kidney sphingosine kinase (Meyer zu Heringdorf et al., 1998, supra), and then incubated for 30 min at 37°C. Labeled SPP was quantified as described above. For each experiment, known amounts of sphingosine were used to generate a standard curve.

Mass measurements of ceramide - Mass amounts of ceramide in cellular extracts were measured by the diacylglycerol kinase enzymatic method. Briefly, an aliquot of the organic phase from cellular lipid extracts or standard bovine brain type IV ceramides were resuspended in 40 μl of 7.5 % (w/v) octyl-β-D-glucopyranoside/5 mM cardiolipin in 1 mM DETPAC/10 mM imidazole (pH 6.6) and solubilized by freeze-thawing and subsequent sonication. The enzymatic reaction was started by the addition of 20 μl DTT (20 mM), 10 μl of *E. coli* diacylglycerol kinase (0.88 U/ml), 20 μl [γ-^{32}P]ATP (10 μCi, 10 mM) and 100 μl reaction buffer (100 mM imidazole (pH 6.6), 100 mM NaCl, 25 mM $MgCl_2$, and 2 mM EGTA). After incubation for 1 h at room temperature, lipids were extracted with 0.5 ml chloroform/methanol/conc. HCl (100:100:1, v/v) and 85 μl of 1 M KCl. Labeled phosphatidic acid and ceramide-1-phosphate were resolved by TLC with

chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v) and quantified with a Molecular Dynamics Storm phosphoimager.

Measurement of total cellular phospholipids Total phospholipids present in cellular lipid extracts were quantified as previously described (Bornfeldt, K. E. et al., 1995, *J. Cell Biol.* 130, 193-206). Briefly, to dried aliquots of cellular lipid extracts, 40 µl of a mixture of 10 N H_2SO_4/60% perchloric acid (1:3, v/v) were added and samples were heated for 30 min at 210°C. After cooling, 75 µl of water and 400 µl of 4.2% ammonium molybdate in 4N HCl/0.045% (w/v) malachite green (1:3 v/v) were added. Samples were incubated overnight at room temperature and absorbances measured at 660 nm.

Northern blotting analysis - Poly(A)$^+$ RNA blots containing 2 µg of poly(A)$^+$ RNA per lane from multiple mouse adult tissues were purchased from Clontech. Blots were hybridized with the 0.8 kb SalI fragment of pCMV-Sport2-mSPHK1, which was gel-purified and labeled with [^{32}P]dCTP by random priming. Hybridization in ExpressHyb™ buffer (Clontech) at 65°C overnight was carried out according to the manufacturer's protocol. Bands were quantified using the Molecular Dynamics PhosphorImager.

Preparation of recombinant sphingosine kinase -HEK293 cells were plated on collagen coated 100 mm dishes and transfected with plasmid vectors containing sphingosine kinase (pCMV-SPORT2sphk1a) as previously described (Kohama et al., 1998, supra). Cells were washed with cold PBS and scraped in Buffer A [200 mM Tris (pH 7.4) containing 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, plus phosphatase inhibitors (1 mM sodium orthovanadate and 15 mM NaF) and protease inhibitors (10 µg/ml leupeptin, 10 µg/ml

aprotinin, and 1 mM PMSF) and the pyridoxal phosphate analog 4-deoxypyridoxine (0.5 mM) to inhibit pyridoxal-dependent SPP lyase]. Cells were then disrupted by freeze-thawing 5 times, centrifuged at 100,000 x g for 60 min and the supernatants stored at 1 mg/ml in storage buffer (Buffer A containing 0.05% Triton X-100 and 10% sucrose and 1 M NaCl) at -80°. Sphingosine kinase activity was stable for several months with no significant loss in activity. Aliquots (10 µg) of recombinant sphingosine kinase from HEK293 cells were used for each SPP or sphingosine mass measurement.

Measurement of SPP

Cells were incubated in low serum or serum-free media for at least 24 h. The media was then removed and cells were washed with PBS and scraped in 1 ml 25 mM HCl/methanol and SPP levels measured as previously described (Kohama et al., 1998, supra). Briefly, lipids from the cells were then extracted with 5 ml of chloroform/methanol/1 M NaCl (2:1:2, v/v) containing 100 µl 3 M NaOH. SPP is water-soluble at alkaline pH, and partitions into the aqueous phase. To the aqueous fraction of lipid extracts (3 ml total), 450 µl of buffer B (200 mM Tris-HCl (pH 7.4), 75 mM $MgCl_2$ in 2 M glycine buffer, pH 9.0) and 50 units of alkaline phosphatase (bovine intestinal mucosa, Type VII-NT) were added, and samples were incubated at 37° for 30 min. To terminate the reaction, 50 µl conc. HCl was added and lipids were extracted twice with 1.5 ml $CHCl_3$. Organic fractions were dried under nitrogen in siliconized glass tubes and resuspended in 175 µl of buffer A containing 0.25 % Triton X-100. Samples were vortexed thoroughly and bath sonicated on ice for 5 min. Recombinant sphingosine kinase (10 µg) was added to the solubilized lipids and reactions started by

addition of 10 µl [g-^{32}P]ATP (10 µCi, 20 mM) containing 200 mM $MgCl_2$. Samples were incubated for 30 min at 37°C and reactions terminated by addition of 50 µl 2N HCl and 0.5 ml $CHCl_3$/methanol/concentrated HCl (100:200:1). Samples were vigorously vortexed, and phases separated by addition of 250 µl $CHCl_3$ and 250 µl 1M NaCl. After centrifugation, an aliquot of the organic phase was separated by TLC on silica gel G60 using $CHCl_3$/methanol/acetone/acetic acid/water (10:5:3:2:1) and visualized and quantified with a a Molecular Dynamics Storm phosphoimager (Sunnyvale, CA), or alternatively, the radioactive spots corresponding to authentic SPP were scraped from the plates and counted in a scintillation counter (Kohama et al., 1998, supra; Olivera and Spiegel, 1998, In Methods in Molecular Biology, vol. 105. I.M.Bird, editor. Humana Press Inc., Totawa, N.J. 233-242). Total phospholipids in cellular lipid extracts were quantified by a colorimetric reaction with malachite green exactly as previously described (Edsall et al., 1997, *J. Neurosci.* 17, 6952-6960).

Measurement of total cellular phospholipids

Total phospholipids in cellular lipid extracts were quantified as previously described with minor modifications (Van Veldhoven, P. P. and Mannaerts, G. P., 1987, *Anal. Biochem.* 161, 45-48). Briefly, to dried aliquots of cellular lipid extracts, 40 µl of a mixture of 10 N H_2SO_4/60% $HClO_4$ (1:3, v/v) were added and samples heated for 30 min at 210°C. After cooling, 75 µl water and 400 µl 4.2% ammonium molybdate in 4 N HCl/0.045% (w/v) malachite green (1:3, v/v) were added. Samples were incubated overnight at room temperature and absorbance measured at 660 nm.

Statistics - Statistics were performed using the students t-test, with values of $p \leq 0.05$ considered significant.

Cloning and expression of sphingosine kinase - SPHK 1a (accession number AF068748) was subcloned into a modified pcDNA3 vector (Invitrogen, Carlsbad, CA) to express proteins with an N terminal c-myc epitope tag (a gift from Dr. Peter Burbelo) by PCR using a 5' primer with a BamH1 restriction site (5'-GAGGGATCCGAACCAGAATGCCCTCGAGGA-3' (SEQ ID NO:12)), and as the 3' primer, the last 21 nucleotides of the SPHK1a sequence with an EcoRI overhang (5'-GAGGAATTCTTATGGTTCTTCTGGAGGTGG-3') (SEQ ID NO:13)). For transient expression, plasmids were transfected into cells using Lipofectamine Plus (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions at a 5:1 ratio with pCEFL GFP, which encodes green fluorescent protein (a generous gift of Dr. Silvio Gutkind). Transfection efficiencies were typically 30% and 40% for NIH 3T3 and HEK293 cells, respectively. Stable transfectants containing pcDNA3 plasmids were selected in medium containing 1 mM sodium pyruvate and 0.5 g/L G418 (NIH 3T3 fibroblasts) or 1 g/L G418 (HEK293 cells).

Measurement of [^{32}P]SPP release - HEK293 cells were grown to subconfluency in 100 mm dishes in 10% FBS media, washed and incubated in serum-free media in containing 40 µCi/ml [^{32}P]orthophosphate for 24 h to label the phospholipid pools to isotopic equilibrium. In some experiments, sphingosine (5 µM) was added to the media 10 min prior to the termination of the 24 h incubation period. The media was then collected and the cells scraped from the dishes. Cellular and secreted [^{32}P]SPP were extracted

in alkaline conditions as described above, followed by acidic extraction with chloroform/methanol/conc. HCl (100:100:1, v/v), to partition [^{32}P]SPP into the organic phase. [^{32}P]SPP was resolved on TLC with 1-butanol/ethanol/acetic acid/water (80:20:10:20, v/v), visualized and quantified as described (Olivera et al., 1998, *J. Biol. Chem.* 273, 12576-12583).

Incorporation of bromodeoxyuridine - 24 h after transfection, NIH 3T3 cells were serum-starved in DMEM supplemented with 2 µg/ml insulin, 2 µg/ml transferrin, and 20 µg/ml BSA, and then stimulated with various agents. After 16 h, cells were incubated for 3 h with bromodeoxyuridine (BrdU) (10 µM), and then fixed in 4% paraformaldehyde containing 5% sucrose (pH 7.0) for 20 min at room temperature. After washing with PBS, cells were incubated in permeabilization buffer (0.5% Triton/PBS pH 7.4 containing 10 mg/ml BSA) for 20 min at room temperature and then incubated for 1 h at room temperature with monoclonal anti-BrdU antibody in the presence of DNAse (1000 U/ml) (Van Brocklyn et al., 1998a, *J. Cell Biol.* 142, 229-240). After washing with PBS, cells were stained with Texas Red-conjugated anti-mouse antibody in 5% BSA/PBS for 1 h, washed with PBS, and then photographed using a Nikon Eclipse TE200 inverted fluorescence microscope connected to a Sony DKC5000 digital camera. Cells expressing GFP and cells with positive BrdU staining were counted. At least three different fields were scored with a minimum of 100 cells scored per field.

Measurement of DNA synthesis - Stably transfected NIH 3T3 fibroblasts were plated in 24 well clusters at a density of $5x10^3$ cells/well in DMEM containing 10% calf serum. After 24 h, cells were washed with DMEM containing 0.5% calf serum and

incubated in same media. The media was replaced every 2-3 days. At the indicated times, cultures were pulsed with 1 μCi of [^{3}H]thymidine for 6 h and radioactivity incorporated into trichloroacetic acid-insoluble material measured as previously described (Olivera and Spiegel, 1993, *Nature* 365, 557-560). Values are the means of triplicate determinations and standard deviations were routinely less than 10% of the mean.

Staining of apoptotic nuclei - Transiently transfected NIH 3T3 cells were cultured in serum-free medium for 1 or 2 days. Cells expressing GFP were examined with an inverted fluorescent microscope. Apoptotic cells were distinguished by condensed, fragmented nuclear regions. The percentage of intact and apoptotic nuclei in cells expressing GFP fluorescence were determined. A minimum of 500 cells were scored. Stably transfected NIH 3T3 fibroblasts were washed and incubated in serum free media containing the indicated agents for 24 h, and stably transfected HEK293 cells were serum-starved for 30 h, washed and incubated in serum free media containing the indicated agents. Cells were then fixed with 3.7% formalin, washed with PBS and treated with bisbenzimide trihydrochloride (24 μg/ml; Hoechst #33258) for 10 minutes. Stained apoptotic nuclei were scored.

Chemotactic motility assay - Boyden chamber chemotaxis assays were carried out essentially as described previously using polycarbonate filters coated with 5 μg of collagen I, which promotes uniform attachment to and migration across the filter without formation of a barrier (Wang et al., 1999, Exp. Cell Res.). Briefly, polycarbonate filters (13 mm diameter, 12 μm pore size) were coated with 5 μg

of collagen I, and then placed into the lower chamber (growth area = 18 mm^2). FBS (10%) or PDGF (20-60 ng/ml) were placed in the lower chamber as chemoattractants. Transfected NIH 3T3 cells were harvested by trypsinization, washed twice with DMEM containing 0.1% BSA, and added to the upper chamber at $5x10^5$ cells per well. The chambers were incubated in a humidified incubator at 37°C in 5% CO_2/95% air for 6 or 24 h, as indicated. The cells which traversed the collagen and spread on the lower surface of the filter were fixed in methanol for 8 min and stained with crystal violet. Nonmigratory cells on the upper membrane surface were removed with a cotton swab. The number of migratory cells per membrane was enumerated using light microscopy at 40X magnification (Wang et al., 1999, supra).

Western blotting - Cytosolic fractions (5-10 µg) were boiled in Laemmli sample buffer and separated by 10% SDS-PAGE. After transferring the protein bands to nitrocellulose membranes, the membranes were blocked with 3% non-fat dry milk in 0.1% Tween-20-PBS, and incubated overnight with anti c-myc monoclonal antibody (1 µg/ml) in the same buffer containing 1% non-fat dry milk. Immunocomplexes were formed using a biotynylated anti-mouse secondary antibody and an avidin-biotinylated horseradish peroxidase complex, and visualized by enhanced chemiluminiscence (ECL) as described previously (Olivera et al., 1997, *Biochim. Biophys. Acta* 1348, 311-323).

Immunostaining - Cells grown on glass coverslips coated with collagen I were incubated overnight in DMEM supplemented with 2 µg/ml insulin, 2 µg/ml transferrin, and 20 µg/ml BSA. Cells were washed with PBS and fixed in 3.7% formalin and 0.1%

Triton X-100 for 20 min. After washing with PBS, cells were permeabilized for 10 min with 0.5% Triton X-100 in PBS, washed again and incubated with anti-myc antibody for 20 min at room temperature . After washing, cells were incubated with anti-mouse antibody conjugated with fluorescein or Texas Red for 20 min. After washing three times with PBS, coverslips were mounted on slides using an Anti-Fade kit (Eugene, OR) and cells were photographed using an inverted fluorescence microscope connected to a Sony DKC5000 digital camera.

Adhesion assay - Collagen I (0.1 mg/ml), fibronectin (0.5 mg/ml), polylysine (0.1 mg/ml), or Matrigel (1:10 dilution) were added into wells of a 6-well culture plate and incubated for 45-60 min at room temperature. Plates were then incubated with 3% BSA in PBS for 30 min to block non-specific binding sites followed by three washes with PBS. NIH 3T3 fibroblasts were harvested by scraping in PBS/10 mM EDTA, washed and resuspended in DMEM/BSA at 10^5 cells/ml, and 2 ml suspensions were then added to each well and incubated at 37°C for the indicated time. Non-adherent cells were removed and attached cells fixed with 70% ethanol for 20 min and stained with crystal violet. Wells were gently rinsed three times with water and allowed to dry. Incorporated dye was dissolved in 100 µl/well of 0.1M sodium citrate in 50% ethanol (pH 4.2) and the absorbance measured at 540 nm (Wang et al., 1999, supra).

Example 1

Sequencing and cloning of sphingosine kinase - The 49 kD sphingosine kinase polypeptide, purified from rat kidney, was excised from an SDS gel and subjected to trypsin digestion. The resulting peptides were separated by microcapillary reverse-

phase HPLC and sequences of 8 peptides were determined by Edman degradation or MALDI mass spectrometry (Table 1). Homology searches (BLAST) against a comprehensive nonredundant database revealed no matches to known proteins. However, when the database of expressed sequence tags (dbEST) at NCBI was searched using the tBLASTn algorithm, an EST (Genbank accession number AA011725) containing sequences homologous to 3 of the 8 peptides (peptides 5, 2, and 4) was retrieved. A further search with peptides 1, 3 and 7 yielded 4 additonal ESTs (Genbank accession numbers AA000819, AA107451, AA592274, and AA389543). The nucleotide sequences of mouse ESTs AA000819 and AA592274 were then used to search dbEST to obtain EST AA389187. Clones AA107451 and AA389187 were highly homologous at their 3′ ends, but were slightly divergent at their 5′ends. Sequencing of the full-length cDNAs revealed apparent open reading frames coding for 381 and 388 amino acid polypeptides containing sequences highly homologous to seven isolated peptides distributed throughout the protein, and these are thus designated SPHK1a and SPHK1b (Fig. 1). In addition, both contained a portion of peptide 8. SPHK1a and 1b have predicted pIs of 6.68 and 6.89 and MWs of 42344 and 43254, respectively, in agreement with the MW of purified rat kidney sphingosine kinase (Olivera et al., 1998, supra). Because SPHK1b only differs by a few amino acids at the N terminus, it may arise by alternative splicing. However, both sequences lacked Kozak concensus sequences, suggesting that these cDNAs may not include the actual initiation sequences..

SPHK1a has 2 overlapping calcium/calmodulin binding concensus sequences of the 1-8-14 Type B motif ((FILVW)xxxxxx(FAILVW)xxxxx(FILVW), containing net positive charges of 2-4) (Rhoads, A. R., and Friedberg,

F., 1997, *FASEB J.* 11, 331-340). In addition, near the C-terminus, SPHK1a contains 2 overlapping calcium/calmodulin binding consensus sequences, one of Type B and one of Type A ((FILVW)xxx(FAILVW)xx(FAILVW)xxxxx(FILVW)) containing net positive charges of 3 - 6) (Rhoads and Friedberg, 1997, supra). SPHK1b contains all the above calcium/calmodulin binding consensus sequences as well as an additional Type B motif at the N-terminus. The existence of multiple calcium/calmodulin binding motifs supports our previous observations that purified rat kidney sphingosine kinase binds tightly to calmodulin-sepharose in the presence of calcium (Olivera et al., 1998, supra).

Analysis of the domain structure of SPHK1a obtained by searching the protein data base (GenBank and Prosite) revealed several putative postranslational phosphorylation motifs: one kinase A, two casein kinase II, and eight protein kinase C phosphorylation sites (Fig. 1). Interestingly, we previously demonstrated that inhibition of ceramide-induced apoptosis by protein kinase C activation results from stimulation of sphingosine kinase and concomitant increase in cellular SPP levels (Cuvillier, O. et al., 1996, *Nature* 381, 800-803). Sphingosine kinase is thought to be mainly a cytosolic enzyme (Olivera et al., 1998, supra). Consistent with this, a hydropathy plot indicates that SPHK1a does not contain signal peptide or hydrophobic transmembrane sequences (data not shown).

Sphingosine kinase appears to be remarkably devoid of recognizable regulatory domains, including SH2, SH3, or PH domains. However, a domain reminiscent of a proline-rich SH3 binding site (Ren, R. et al., 1993, *Science* 259, 1157-1161) is present at

the N-terminus. Furthermore, there were no obvious similarities between sphingosine kinase and phosphatidylinositol-3 kinase or phosphatidylinositol-4 kinase. Previously, we have shown that sphingosine kinase is activated by acidic phospholipids including phosphatidic acid, phosphatidyl inositol, phosphatidyl inositol 4,5-bisphosphate, and particularly phosphatidyl serine, yet bears no similarity to proteins known to bind these phospholipids, such as phosphatidic acid phosphohydrolase, phospholipase C, or protein kinase C. On the other hand, a data base search identified homologs of sphingosine kinase in numerous widely disparate organisms, demonstrating that sphingosine kinase is a member of a novel but highly conserved gene family (Fig. 2). Two of these genes, named LCB4 and LCB5, were recently shown to code for sphingosine kinases from *Saccharomyces cerevisiae* (Nafiec, M. M. et al., 1998, J. Biol. Chem. 273, 19437-19442). Most of the other cDNA sequences were from ESTs or putative open reading frames identified from *C. elegans* and *S. pombe* genomic sequences. A comparison of these sequences reveals the location of several blocks of highly conserved amino acids, one or more of which might constitute critical portions of catalytic or substrate binding sites. Five regions, in particular (C1 to C5), are highly conserved in all of these sequences. C1 contains an invariant positively charged motif, GGKGK, which may be part of the ATP binding site. Moreover, the C1 and C3 subdomains of sphingosine kinase show high amino acid similarity to residues 296-315 and 378-389 of human diacylglycerol kinase ζ with 35% and 58% identity, respectively (Bunting, M. et al., 1996, *J. Biol. Chem.* 271, 10230-10236; Ding, L. et al., 1997, *Proc. Natl. Acad. Sci. U.S.A.* 94,

5519-5524). These residues are present in subdomain 1 (C4-a) which is conserved in all of the known diacylglycerol kinase family members (Klauck, T. M. et al., 1996, *J. Biol. Chem.* 271, 19781-19788; Tang, W. et al., 1996, *J. Biol. Chem.* 271, 10237-10241). Although subdomain 1 in diacylglycerol kinases contains a GXGXXG box which is similar to the $GXGXXGX_{12-14}K$ motif, known to participate in ATP binding to protein kinases, the downstream K is missing. However, the GXGXXG box (Sakane, F. et al., 1990, *Nature* 344, 345-348) is not present in sphingosine kinase. Furthermore, recent studies demonstrated that this box may not be required for diacylglycerol kinase activity (Schaap, D. et al., 1995, *Biochem. J.* 304, 661-664).

Example 2

Tissue distribution of sphingosine kinase

The tissue distribution of sphingosine kinase mRNA expression in adult mouse tissues was analyzed by Northern blotting (Fig. 3). In most tissues, including adult brain, heart, spleen, lung, kidney, and testis, a predominant 2.4 kb mRNA species was detected, indicating ubiquitous expression of sphingosine kinase. However, the level of expression was markedly variable among the different tissues. The mRNA levels were highest in adult lung and spleen and there were barely detectable levels in skeletal muscle and liver. The mSPHK1 mRNA detected by Northern blotting was slightly larger than the size of the cDNA (1.9 kb). Interestingly, expression of mSPHK1a mRNAs in various mouse tissues did not closely correlate with the relative sphingosine kinase activities in rat tissues, as we previously found that spleen and kidney have higher specific activities than

liver, which has about twice the activity of brain (Olivera et al., 1998, supra).

Example 3

Recombinant sphingosine kinase activity

To investigate whether SPHK1a and SPHK1b encode bona fide sphingosine kinases, HEK293 cells were transiently transfected with pCMVSPORT2 expression vectors containing either SPHK1a or SPHK1b cDNAs and sphingosine kinase activity was measured. Modest levels of endogenous sphingosine kinase activity were present in control cells (either untransfected or transfected with an empty vector) (Fig. 4). Cells transfected with SPHK1a exhibited 300-fold increased sphingosine kinase activity 24 h after transfection that remained at this level for 4 days. In contrast, cells transfected with SPHK1b showed only 120 fold increased sphingosine kinase activity after 24 h and then decreased gradually to control levels after 4 days (Fig. 4). Transfection of either Swiss 3T3 or NIH 3T3 fibroblasts with SPHK1a or SPHK1b also resulted in marked increases in sphingosine kinase activity (Table II). As in HEK293 cells, transfection of 3T3 cells with SPHK1a led to much larger increases in sphingosine kinase activity than with SPHK1b. It should be noted that transfection efficiency was quite good and similar in all three cell lines (Table II).

TABLE II. SPECIFIC ACTIVITY OF MURINE SPHINGOSINE KINASE IN TRANSIENTLY TRANSFECTED CELL LINES

Cells	DNA	Activity pmol/min/mg protein	Activity fold increase	Transfection efficiency
HEK293	Control	29	1.0	32.2
	SPHK1a	23,200	800	42.0
	SPHK1b	657	22.5	45.5
NIH 3T3	Control	31	1.0	33.7
	SPHK1a	12245	395	ND
	SPHK1b	167	5.4	28.5
Swiss 3T3	Control	6.2	1.0	38.1
	SPHK1a	3200	516	ND
	SPHK1b	15.7	2.5	42.1

Various cells lines were transfected with vector alone (pCMVSPORT2) or with expression vector containing either SPHK1a or SPHK1b cDNA. Transfection efficiency was determined by co-transfection with pCEFLGFP and enumeration of cells expressing green fluorescence. Sphingosine kinase activity in cell lysates was measured as described under "Experimental Procedures". Results are representative of three independent experiments. ND, not determined.

Cells transfected with either SPHK1a or SPHK1b exhibited substantial increases in cytosolic (870 and 17 fold, respectively) and membrane-associated (800 and 45 fold, respectively) sphingosine kinase activity (Fig. 5). The relative amounts of sphingosine kinase activity in cytoplasmic versus membrane fractions were similar in vector-transfected and SPHK-transfected

cells. Both membrane-associated and cytosolic sphingosine kinase activities have been described in mammalian tissues and cell lines (Olivera and Spiegel, 1993 supra; Olivera et al., 1994a, supra; Stoffel, W. et al., 1973, *Hoppe-Seyler's Z. Physiol. Chem.* 354, 1311-1316; Ghosh, T. K. et al., 1994, *J. Biol. Chem.* 269, 22628-22635) and it has been suggested that these activities might have distinct physiological roles and might be derived from different gene products. In contrast, we demonstrate here that sphingosine kinase expressed from a single gene product is present both in the cytoplasm and in membranes.

The substrate specificity of sphingosine kinase activity from cells transfected with either SPHK1a or SPHK1b was the same as found previously for purified rat kidney sphingosine kinase (Olivera et al., 1998, supra). The naturally occurring D-(+)-*erythro*-trans-isomer was the best substrate. However, D,L-*erythro*-dihydrosphingosine was phosphorylated to a similar extent, whereas, phyto-sphingosine, D,L-*threo*-dihydrosphingosine, ceramide, diacylglycerol, and phosphatidylinositol were not phosphorylated (Fig. 6A). Typical Michaelis-Menten kinetics were observed with D-*erythro*-sphingosine with Km values of 2.15 μM (data not shown), in excellent agreement with values found with sphingosine kinase purified to homogeneity from rat kidneys (Olivera et al., 1998, supra). N,N-dimethylsphingosine and D,L-*threo*-dihydrosphingosine have previously been used to inhibit sphingosine kinase and decrease SPP levels stimulated by various physiological stimuli (Olivera and Spiegel, 1993, supra; Meyer zu Heringdorf, et al., 1998, supra; Cuvillier, O. et al., 1996, supra). Both of these sphingolipids were potent competitive inhibitors of sphingosine kinase from transfected cells (Fig. 6B,C).

Transfection of NIH 3T3 cells with SPHK1a also resulted in marked changes in mass levels of sphingolipid metabolites (Fig.7). Mass levels of SPP increased 21-fold compared to cells transfected with vector alone, with a concomitant 67% decrease in levels of sphingosine and, to a lesser extent, in ceramide levels (29%). However, it should be noted that the absolute decrease in mass of ceramide was actually much greater than the decrease in mass of sphingosine. Our results suggest that transfected SPHK1a is active in intact cells, and overexpression alters the balance of sphingolipid metabolites within cells.

Example 4

Characterization of cells expressing sphingosine kinase - Similar to our previous results with transiently transfected cells (Kohama et al., 1998, supra), SPHK activity in NIH 3T3 and HEK293 cells stably expressing c-myc tagged SPHK1a was dramatically increased by 500 fold (Fig. 8A). Western blot analysis of cytosolic fractions using anti-c-myc antibody revealed a specific protein band with an apparent molecular weight consistent with the predicted size of c-myc-SPHK which was absent in vector transfected cells. SPP levels were also elevated in cells expressing SPHK (Fig. 8B), although the increases were only 4 -8 fold and did not correlate with the large fold increase in SPHK activity measured *in vitro*. One possible explanation for this discrepancy is that availability of cellular sphingosine might limit the production of SPP. In agreement with our previous results (Zhang et al., 1991, *J. Cell Biol.* 114, 155-167), when cells were

acutely treated with exogenous sphingosine, which is readily taken up (Olivera et al., 1997, *Biochim. Biophys. Acta*. 1348, 311-323; Olivera et al., 1994a, supra; Olivera et al., 1994b, *J. Biol. Chem.* 269, 17924-17930), levels of SPP were further increased 3-6 fold, suggesting that although availability of sphingosine may be important for regulating SPP levels, it is probably not the only critical factor influencing levels of SPP in cells overexpressing SPHK.

Since activated platelets can release SPP (Yatomi et al., 1995, *Blood* 86, 193-202), and we recently have identified a family of G protein-coupled SPP receptors (Lee et al., 1998, *Science* 279, 1552-1555; Van Brocklyn et al., 1998a, *J. Cell Biol.* 142, 229-240), it was important to determine whether SPHK-transfected cells, which have notable increases in SPP levels, secrete SPP into the medium. No significant release of SPP into the extracellular media could be detected, even after addition of sphingosine. To increase the sensitivity of detection of secreted SPP, we labeled cells to isotopic equilibrium with [^{32}P]P_i and analyzed the labeled SPP in cells as well as in the medium (Fig. 8C). Despite the large increases in [^{32}P]SPP detected in cells overexpressing SPHK, there was no detectable labeled SPP released into the medium. Both SPP assays gave identical increases in intracellular SPP in transfected and sphingosine-treated cells. Based on the sensitivity of these methods (1-2 pmol of SPP/sample), it is estimated that the concentration of SPP in the extracellular media must be less than 0.4 nM, a concentration well below the Ec50 for

binding of SPP to its receptors (Van Brocklyn et al., 1998, supra; Van Brocklyn et al., 1999, supra).

Example 5

Localization of sphingosine kinase and stimulation by growth factors - Both membrane-associated and cytosolic SPHK activities have been described in mammalian tissues and cell lines (Buehrer and Bell, 1992, *J. Biol. Chem.* 267, 3154-3159; Ghosh et al., 1994, supra; Olivera et al., 1994a, supra; Olivera and Spiegel, 1993, supra; Stoffel et al., 1973, *Hoppe-Seyler's Z. Physiol. Chem.* 354, 1311-1316). However, the amino acid sequence of murine SPHK1a suggests that it should be a cytosolic protein (Kohama et al., 1998, supra). In agreement with our previous study (Kohama et al., 1998, supra), most of the SPHK activity in cells stably expressing c-myc-tagged SPHK was cytosolic (data not shown), suggesting that the small c-myc-tag does not affect localization of SPHK. Immunohistochemistry with antibodies against c-myc revealed that SPHK has a diffuse distribution in the cytosol and somewhat denser expression in perinuclear sites (Fig. 9).

Previously, we and others have shown that PDGF stimulates SPHK activity in various cell types (Bornfeldt et al., 1995, supra; Olivera and Spiegel, 1993, supra; Pyne et al., 1996, *Eur. J. Biochem.* 237, 819-826; Rani et al., 1997, supra). PDGF stimulated cytosolic c-myc-SPHK activity in transfected NIH 3T3 fibroblasts to a similar extent as its effect on endogenous SPHK (Table 3), indicating that c-myc-SPHK activity is regulated by the signaling pathways triggered by growth factors in the same manner as the native enzyme. Collectively, these data suggest that

cells overexpressing SPHK are a useful tool to study intracellular actions of SPP.

TABLE 3. PDGF STIMULATES SPHINGOSINE KINASE ACTIVITY IN TRANSFECTED CELLS

	SPHINGOSINE KINASE ACTIVITY (pmol/min/mg)	
	Vector	SPHK
treatment		
None	68	5900
PDGF (1 ng/ml)	86	7992
PDGF (20 ng/ml)	124	9092

NIH 3T3 cells were transfected with c-myc-pcDNA3-SPHK1a or c-myc-pcDNA3, incubated in serum-free media containing fatty acid free BSA (20 µg/ml), transferrin (2 µg/ml) and insulin (2 µg/ml) for 18 h, and then stimulated with the indicated concentrations of PDGF for 10 min. Cytosolic SPHK activity was measured as described in Materials and Methods and standard deviations were <10%.

Example 6

Effect of sphingosine kinase overexpression on cell proliferation - The most well established biological response to SPP is the stimulation of cell growth (Blakesly et al., 1997, *J. Biol. Chem.* 272, 16211-16215; Gomez-Munoz et al., 1995, *J. Biol. Chem.* 270, 26318-26325; Goodemote et al., 1995, *J. Biol. Chem.* 270, 10272-10277; Olivera and Spiegel, 1993, supra; Pyne et al., 1996, supra; Rani et al., 1997, supra; Su et al., 1994, *J. Biol.*

Chem. 269, 16512-16517; Wu et al., 1995, *J. Biol. Chem.* 270, 11484-11488; Zhang et al., 1991, supra), and ample evidence suggests that this is due to its intracellular actions (Olivera and Spiegel, 1993, supra; Van Brocklyn et al., 1998, supra). Thus, it was of interest to examine the proliferation of cells whose endogenous levels of SPP are increased after transfection with SPHK. Transient expression of c-myc-SPHK in NIH 3T3 cells not only increased intracellular levels of SPP, it also increased the proportion of cells in S phase measured as incorporation of BrdU into nascent DNA (Fig. 10A). Moreover, serum and PDGF potentiated the growth promoting effects of SPHK (Fig. 10A). However, addition of exogenous SPP increased BrdU incorporation in empty vector-transfected cells by 2 fold, although it had no significant mitogenic effect on SPHK-transfected cells, suggesting that these cells are already maximally stimulated by their endogenous SPP.

Since transient expression of SPHK increased the proportion of cells in the S phase, it was of interest to determine whether there was a corresponding increase in cell number as a result of stable transfection. Stable expression of SPHK had a dramatic effect on the growth of these cells in low serum media (Fig. 11). While empty vector-transfected cells grew very slowly over the course of 8 days in 0.5% serum, overexpression of SPHK was sufficient to confer serum-independent growth and these cells reached confluency after 6 days in low serum (Fig. 11).

Example 7

Effect of sphingosine kinase overexpression on programmed cell death - SPP has been shown to suppress apoptosis induced by cytokines, such as TNF and Fas ligand (Cuvillier et al., 1996, supra), serum deprivation (Edsall et al., 1997, supra; Van Brocklyn et al., 1998, supra), and to be important in the survival effects of NGF (Edsall et al., 1997, supra), vitamin D3 (Kleuser et al., 1998, *Cancer Res.* 58, 1817-1824) and cAMP (Machwate et al., 1998, *Mol. Pharmacol.* 54, 70-77). If the intracellular level of SPP is a critical factor that determines cell survival, then it is expected that overexpression of SPHK should suppress apoptosis. Serum deprivation induced apoptosis in NIH 3T3 fibroblasts, where shrinking, blebbing, and condensation of nuclei were clearly evident after 24 h. Transient expression of SPHK in NIH 3T3 fibroblasts suppressed the appearance of apoptotic nuclei induced by serum starvation (Fig. 12A). It should be pointed out that serum withdrawal markedly increases ceramide levels, and it has been proposed that ceramide mediates, at least in part, serum deprivation-induced cell death (Hannun, 1996, *Science* 274, 1855-1859). Similar anti-apoptotic effects were observed in NIH 3T3 fibroblasts and HEK293 cells stably expressing SPHK (Fig. 12B-D). Expression of SPHK in HEK293 cells also inhibited apoptosis induced by the cell permeable ceramide analog, C2-ceramide, but to a lesser extent (Fig. 12D). Furthermore, transient expression of SPHK in U937 cells reduced apoptosis induced by both serum starvation and TNF-α treatment, as determined by DNA fragmentation and DEVDase activity measurements with Ac-DEVD-AMC as a

substrate (data not shown). In contrast, SPHK expression had almost no effect on apoptosis resulting from treatment with staurosporine, a broad spectrum protein kinase inhibitor which is known to induce apoptosis in normal and neoplastic cells (Jacobsen et al., 1996, *J. Cell Biol.* 133, 1041-1051). Further experiments revealed that although SPHK does not contain the $GXGXXGX_{12-14}K$ motif, known to participate in ATP binding to protein kinases, it is inhibited by staurosporine *in vitro* (data not shown). Thus, the presence of high levels of SPP in various cell types due to overexpression of SPHK, can mimic the protective effect of exogenous SPP against apoptosis, specially in response to growth factor withdrawal.

Example 8

Effect of sphingosine kinase overexpression on chemotaxis - Previously, many studies have shown that exogenous SPP inhibits chemotactic and haptotactic motility of various cancer cells by binding to putative cell surface receptors (Kawa et al., 1997, *FEBS Lett.* 420, 196-200; Sadahira et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89, 9686-9690; Sadahira et al., 1994, *FEBS Lett.* 340, 99-103). Furthermore, Yamamura et al. found that low nanomolar concentrations of SPP and SPP immobilized on controlled pore glass beads inhibited the motility of mouse melanoma B10 cells (Yamamura et al., 1997, *Biochemistry* 36, 10751-10759). Thus, it was expected that overexpression of SPHK, which increases endogenous, but not secreted SPP (Fig. 8), should not affect cellular movement. Surprisingly, chemotaxis directed towards concentration gradients of PDGF and serum was markedly reduced in NIH 3T3 (Fig. 13) and HEK293 cells (data not shown) transiently or stably overexpressing SPHK. Although chemotaxis measurements

were routinely performed over a 24 h period with a potent chemoattractant such as PDGF, the inhibitory effect of SPP was discernible as soon as 6 h (Fig. 13A). Moreover, expression of SPHK also inhibited chemokinesis, or random cell motility, albeit to a lesser extent (Fig. 13B). To determine whether the effect of SPHK on motility was due to altered adhesion to the collagen I-coated filters used in the Boyden chamber assay, adherence to collagen I was determined. The time-course of adhesion to plastic (not shown) or collagen I coated-wells was similar for vector and SPHK-transfected cells (Fig. 13C). Overexpression of SPHK had no significant effects on the adhesiveness of cells not only to collagen, but also to fibronectin, Matrigel, and poly-lysine (Fig. 13D). Thus, intracellular SPP, rather than secreted SPP, regulates chemotactic motility without affecting adhesion of cells to their substratum.

Example 9

Isolation, detection and quantification of SPP - Conditions were optimized to obtain the highest recovery of SPP during the extraction and enzymatic reactions. Extraction and dephosphorylation efficiencies were monitored with [^{32}P]SPP or [^{3}H]sphingosine. SPP partitions into the aqueous phase at alkaline pH with high recovery (Van Veldhoven, et al., 1994, supra; Yatomi, et al., 1995, supra) with the added advantage of excluding the majority of cellular phospholipids and sphingolipids, including sphingosine, which remain in the organic phase. Extraction of NaOH afforded the highest recovery of SPP into the alkaline aqueous/methanolic phase (90-95%), even in the presence of cellular lipids from serum and tissues which can interfere with extraction. In contrast, lower recovery (<85%) of SPP

was observed when concentrated ammonium hydroxide or 0.4 M borate buffer was used for alkaline extraction in the presence of cellular lipids. Sphingosine (>99%) remained in the organic phase, in agreement with previous reports.

Alkaline extracted SPP was rapidly and reproducibly converted to sphingosine after digestion with bovine intestinal mucosa alkaline phosphatase. We found that more than 85% of added [^{32}P]SPP was converted to sphingosine. Alkaline phosphatase treatment most effectively cleaved the phosphate group from SPP, which was remarkably resistant to acidic or alkaline hydrolysis. Less than 20% hydrolysis of [^{32}P]SPP occurred even after 4 h at 70 °C with 1 M KOH or 1 M HCl. After dephosphorylation, sphingosine is much more soluble in organic solvents than its phosphorylated precursor and in agreement with previous reports, 85-90% of [^{3}H]sphingosine was recovered in the organic layer (Van Veldhoven et al., 1994, supra; Olivera et al., 1994a, supra).

The substrate selectivity of recombinant sphingosine kinase makes our enzymatic method much more specific than other procedures (Van Veldhoven, et al., 1994, supra; Yatomi, et al., 1995, supra) which non-selectively modify the amino group of this sphingoid base. Using our procedure, the ^{32}P-labeled product detected by TLC had the same mobility as standard SPP (Fig. 14A). The amount of [^{32}P]SPP formed was proportional to the amount of SPP standard added over a wide concentration range, from 250 fmol to 2500 pmol (Fig. 14B and data not shown). Presence of different cell lipid extracts did not significantly affect the recovery of [^{32}P]SPP (Fig. 14B, Insert). Moreover, the amount of SPP recovered was proportional to the number of cells extracted (Fig. 14C). The

overall recovery of SPP was approximately 75%.

Example 10

SPP in serum - Because previously it has been reported that serum contains high levels of SPP (Yatomi, Y. et al., 1997, *J. Biochem.* 121, 969-973), we measured SPP levels in serum from various animals (Fig. 15). Indeed, SPP was readily detected in serum, the highest levels occurring in horse serum (0.6 μM), with lesser amounts in fetal bovine serum and calf serum where the concentration is similar to that previously found in human serum (Yatomi et al., 1997, supra). To verify that the presence of sphingosine did not interfere with SPP measurements, SPP in lipid extracts from horse serum and FBS was measured before and after alkaline phosphatase treatment (Fig. 15A). No SPP was detected without alkaline phosphatase digestion, indicating that carryover of sphingosine during lipid extraction was negligible. We found that serum contains sphingosine at concentrations of approximately 0.05-0.1 μM (data not shown). Negligible amounts of SPP were found in calf serum which was charcoal stripped to remove endogenous SPP (Fig. 15B). Hence, we suggest that in order to examine the role of SPP as a ligand for G-protein coupled receptors, cells should be cultured in media containing charcoal-stripped serum.

Example 11

Mass level determination of SPP and sphingosine in rat tissues - Several methods have previously been used to measure the sphingosine and SPP content of rat tissues (Kobayashi, T. et al., 1988, *Eur. J. Biochem.* 172, 747-752; Merrill, A. H. et al., 1988, *Anal. Biochem.* 171, 373-381; Yatomi, Y. et al., 1997, *FEBS Lett.* 404, 173-4). In alkaline extractions, SPP and sphingosine partition into

different phases, thus enabling us to concurrently determine the levels of these sphingolipid metabolites. As shown in Fig. 16A, we found that rat spleen contains the highest level of sphingosine. Moreover, the sphingosine levels in spleen, kidney and liver were in excellent agreement with previously reported mass levels determined by either HPLC, mass spectrometry, or by our previous enzymatic method (Van Veldhoven and Mannaerts, 1987, supra; Merrill et al., 1988, supra; Van Veldhoven, P. P. et al., 1989, *Anal. Biochem.* 183, 177-189).

The highest content of SPP was found in rat brain and spleen, amounts that were similar to those found previously by other methods (Van Veldhoven et al., 1994, supra; Yatomi et al., 1997, supra, 35). A significant amount of SPP was also found in whole rat eye (Fig. 16A). When mass levels of SPP in whole rat eye were normalized to total cellular phospholipid, the abundance of SPP in this tissue was even more evident. In contrast to the results of Yatomi et al who detected large amounts of SPP in testes (Yatomi et al., 1997, supra), we found that testes contain only a relatively small amount of SPP, similar to the level in liver and heart (Fig. 16A). It is interesting to note that in tissues with the highest SPP content, such as brain and eye, the ratio of sphingosine to SPP is approximately 2:1. This contrasts with the other tissues examined in which sphingosine levels are nearly 10-20 fold greater than SPP.

Example 12

SPP and sphingosine in cultured cells -

While sphingosine levels have been determined in a number of cell types (Olivera et al., 1994a, supra;, Van Veldhoven et al., 1989, supra; Wilson, E. et al., 1988, *J. Biol. Chem.* 263, 9304-9309; Ohta, H. et al., 1994, *Anal. Biochem.* 222, 489-494), there have only been scattered reports of SPP measurements. Recently,

a combination of liquid chromatography/ionspray ionization tandem mass spectrometry was used to analyze levels of sphingolipid metabolites in human promyelotic HL-60 cells (Mano et al., 1997, supra). To further establish the validity of the method, we also measured levels of SPP in these cells. Using the recombinant sphingosine kinase method, we obtained values of 2.50 ± 0.281 and 0.112 ± 0.006 pmol/10^6 cells, respectively, in excellent agreement with previous results (Mano et al., 1997, supra; Van Veldhoven et al., 1989, supra). Levels of SPP were also measured in several other cultured cell lines (Table 4). Highest levels of SPP were found in U937 and C6 glioma cells, with values of approximately 2 pmol/10^6 cells, while Jurkat cells only contained 37 fmol/10^6 cells. Like SPP, sphingosine content also varied widely in various cell types, from 0.6-12 pmol/10^6 cells. In all of the cell types examined, except U937 cells, levels of sphingosine were much higher than those of SPP. Although levels of sphingosine in various cell types correlated closely with previous reported values (Mano et al, 1997, supra; Olivera et al., 1994a, supra; Van Veldhoven et al., 1989, supra; Ohta et al., 1994, supra), levels of SPP determined in U937, HL60 and PC12 cells were only 1/10 of those obtained using the method of Yatomi et al (Edsall, L. C. et al., 1997, *J. Neurosci.* 17, 6952-6960; Kleuser, B. et al., 1998, *Cancer Res.* 58, 1817-1824; Cuvillier, O. et al., 1996, *Nature* 381, 800-803; Sato, K. et al., 1997, *Biochem. Biohpys. Res. Commun.* 240, 329-334; Yatomi, T. et al., 1995, *Anal. Biochem.* 230, 315-320). It is not clear why our method yields such different results. It is possible that, in addition to SPP, other lysolipids containing amino groups may also be present in alkaline lipid extracts

and would be detected by the method of Yatomi et al (Yatomi et al., 1995, supra) leading to overestimation of SPP. In contrast, since sphingosine kinase selectively phosphorylates only D-*erythro* sphingosine, and to a lesser extent D-*erythro* dihydrosphingosine, the values obtained with our enzymatic method more likely represent true endogenous levels of this sphingoid base.

TABLE 4. LEVELS OF SPHINGOLIPIDS IN DIFFERENT CELL TYPES

Cells	SPP (pmol/10^6 cells)	Sphingosine (pmol/10^6 cells)	Phospholipids (nmol/ 10^6 cells)
HL60	0.11 ± 0.01	2.50 ± 0.28	8.64 ± 0.98
Jurkat	0.04 ± 0.01	0.61 ± 0.06	3.42 ± 0.12
U937	2.03 ± 0.16	1.38 ± 0.12	8.23 ± 0.56
C6 glioma	1.67 ± 0.39	4.14 ± 0.86	30.9 ± 0.68
MCF7	1.14 ± 0.14	11.0 ± 0.57	23.0 ± 0.12
PC12	0.86 ± 0.06	12.1 ± 0.71	61.0 ± 5.04

SPP, sphingosine, and phospholipids were measured as described in Materials and Methods. The data are the means ± SD of triplicate cultures. Similar values were obtained in five independent experiments.

Example 13

Changes in levels of SPP in PC12 cells after treatment with exogenous sphingosine or NGF - Because exogenous sphingosine has previously been shown to be taken up by various cell types, including PC12 cells, resulting in an its rapid phosphorylation to SPP, it was of interest to confirm

these results using our enzymatic assay. When PC12 cells were treated with 1 μM sphingosine, cellular levels of SPP increased nearly 10-fold within 20 min. These levels were maintained for 1 h and decreased thereafter (Fig. 17).

Sphingosine kinase activity in rat pheochromocytoma PC12 cells has previously been shown to be greatly increased in response to NGF (Edsall et al. 1997, supra). After treatment with NGF, significant increases in SPP levels were detected after 1 h (Fig. 17), with similar fold increases as previously determined by a different method (Edsall et al., 1997, supra). Furthermore, in agreement with our previous results (Edsall et al., 1997, supra; Rius, R. A. et al., 1997, *FEBS Lett.* 417, 173-176), the effect of NGF on sphingosine kinase was biphasic, with greater increases detected after 20 h.

A useful enzymatic assay to measure SPP and sphingosine has been developed which utilizes recombinant sphingosine kinase. The SPP assay described here is similar to our previous method (Olivera et al., 1994a, supra; Olivera and Spiegel, 1998, supra) in which sphingosine kinase in fibroblast extracts was used to determine mass levels of sphingosine. To quantitatively determine cellular levels of SPP using the sphingosine kinase assay, it is necessary to first separate SPP from sphingosine and other cellular lipids, which can be effectively done using the alkaline lipid extraction procedure described here. A major advantage of this assay is that due to the high degree of specificity of sphingosine kinase, D-*erythro* SPP levels can selectively be measured in lipid extracts. Furthermore, this method is highly sensitive and can be used to measure SPP and sphingosine levels that

could only previously be measured using mass spectrometry or HPLC. Since levels of SPP in cultured cells are extremely low (0.1-1 pmol/ 10^6 cells), our method to determine levels of this important sphingolipid second messenger should be very useful. For example, we were able to rapidly and accurately measure basal levels of SPP in various cell types, tissues, and serum, and to quantitate increases in SPP induced by exogenous addition of sphingosine, or by NGF activation of sphingosine kinase in PC12 cells.

DISCUSSION

The complexity of the biological functions of SPP emphasizes the importance of SPHK, the key enzyme responsible for the formation of SPP, whether it functions inter or intracellularly. Activated platelets release intracellularly stored SPP, in a PKC-dependent manner (Yatomi et al., 1997b, *J. Biol. Chem.* 272, 5291-5297). However, we found that several SPHK overexpressing cell lines, including NIH 3T3 fibroblasts and HEK293 cells, do not secrete SPP although they have markedly increased intracellular SPP levels, suggesting that only certain cell types are capable of secreting SPP and that these transfected cells are useful tools to study the intracellular roles of SPP.

SPHK activity and SPP levels can be regulated by external signals (Spiegel et al., 1998a, *Biochemistry (Mosc)* 63, 69-73; Spiegel and Merrill, 1996, *FASEB J.* 10, 1388-1397). Interestingly, the levels of SPP in cells overexpressing SPHK, despite the enormous increases in activity, were maximally increased only 4-8 fold. This level was similar to the level of SPP produced in the same parental cells after stimulation by growth factors, addition of

sphingosine, or even SPP itself (Olivera and Spiegel, 1993, supra). These observations suggest that cells tightly regulate their levels of SPP, consistent with its role as a second messenger. In addition to SPHK, rapid degradation by SPP lyase and/or SPP phosphatase may also play an important role in determining the steady state levels of SPP. Another possible explanation for the lack of correlation of SPHK activity and SPP levels is that the substrate for the overexpressed SPHK, sphingosine, may be located in a different subcellular compartment. Indeed, SPHK is mainly cytosolic, whereas sphingosine is mainly membrane associated. However, availability of sphingosine may not be the only limiting factor for the production of SPP, because addition of exogenous sphingosine increased synthesis of SPP in parental cells and in cells expressing SPHK by the same proportion.

SPHK activity has been found in the cytosolic fraction of most cells [Olivera, 1993 #700; Louie, 1976 #2138; Hirschberg, 1970 #2438; Stoffel, 1973 # 2450] and in the membrane fraction of certain tissues and organs (Buehrer and Bell, 1992, supra; Keenan, 1972, *Biochim. Biophys. Acta.* 270, 383-396; Nagiec et al., 1998, *J. Biol. Chem.* 273, 19437-19442). Recently, it has been reported that human platelets express multiple forms of SPHK differing in their sensitivity to β-octylglucopyranoside and the competitive inhibitors N,N-dimethylsphingosine and L-*threo*-dihydrosphingosine. Only one of these forms is loosely associated with membranes, while two others are cytosolic (Banno et al., 1998, *Biochem. J.* 335, 301-304). Using a c-myc-epitope tag, we examined the subcellular localization of

SPHK by immunohistochemistry. SPHK was expressed diffusely in the cytosol of several types of cells, with denser expression in perinuclear sites. Our observation that SPHK activity in naïve and in SPHK-transfected cells is stimulated to the same extent by PDGF, suggests that the cytosolic form of SPHK is involved in the endogenous production of SPP in response to growth factors and might play an important role in signal transduction pathways.

SPP has been proposed as an intracellular mediator of cell growth (Spiegel and Merrill, 1996, supra), and in agreement, microinjection of SPP (Van Brocklyn et al., 1998, supra) and caged SPP (Qiao et al., 1998, *Bioorg. Med. Chem.* 8, 711-714) is mitogenic for Swiss 3T3 fibroblasts. However, the proliferative response to exogenously added SPP is partially sensitive to pertussis toxin, suggesting the potential involvement of cell surface receptors (Goodemote et al., 1995, *J. Biol. Chem.* 270, 10272-10277). Here, we have shown that cells containing higher levels of SPP due to overexpression of SPHK, not only had a higher proportion in the S phase of the cell cycle but also acquired the characteristic of serum-independent growth, indicating that intracellular SPP is an important regulator of cell growth. Similar conclusions were reached by previous observations in Swiss 3T3 cells using competitive inhibitors of SPHK, where SPP was shown to mediate part of the proliferative signals elicited by PDGF (Olivera and Spiegel, 1993, supra), including activation of mitogen-activated protein kinase, cyclin-dependent kinases (cdc2 and Cdk2 kinases), and activation of the transcription factor AP1 (Rani et al., 1997, supra; Su et al., 1994, supra).

The effects of SPP on cell growth appear to be evolutionarily conserved, as SPP also regulates proliferation and survival of yeast. Spontaneous mutants of *S. cerevisiae* with diminished SPHK activity had reduced growth rates (Lanterman and Saba, 1998, *Biochem. J.* 332, 525-531), suggesting that the evolutionary conserved SPHK (Kohama et al., 1998, supra; Nagiec et al., 1998, supra) also has a conserved function in cell growth. Furthermore, exogenously added SPP was unable to affect yeast growth, suggesting that only accumulation of intracellular SPP is responsible for these effects (Lanterman and Saba, 1998, supra).

Mammalian cells overexpressing SPHK not only had higher growth rates, but also exhibited enhanced survival in serum-free conditions. We have previously shown that SPP can antagonize apoptosis mediated by a number of stress stimuli, including serum withdrawal, TNF-α, Fas ligation, and ceramide elevation (Cuvillier et al., 1996, supra; Edsall et al., 1997, supra). Previous evidence indicated that these effects were not related to cell surface binding (Van Brocklyn et al., 1998, supra). In agreement, our present results indicate that the cytoprotective actions of SPP are mediated intracellularly. Because the sphingolipid metabolite ceramide is a mediator of stress responses (Hannun, 1996, supra), both in mammalian cells and in yeast (Dickson et al., 1997, *J. Biol. Chem.* 272, 30196-30200; Jenkins et al., 1997, *J. Biol. Chem.* 272, 32566-32572; Nickels and Broach, 1996, *Genes Dev.* 10, 382-394), we proposed that the relative intracellular levels of these two sphingolipid metabolites (ceramide and SPP) is an important factor that determines whether cells will survive or die

(Cuvillier et al., 1996, supra; Mandala et al., 1998, *Proc. Natl. Acad. Sci. USA* 95, 150-155). Consistent with this hypothesis, we found that transient expression of SPHK modified the sphingolipid metabolite balance, resulting in higher levels of SPP, with concomitant decreases in the levels of sphingosine and ceramide (Kohama et al., 1998, supra). Our present results show that transient or stable expression of SPHK suppressed apoptosis induced by serum deprivation, known to increase ceramide levels, or by the ceramide analog, C2-ceramide. It is interesting to note that factors that promote both growth and survival, such as PDGF and NGF, stimulate SPHK, suggesting the importance of SPP as part of their signal transduction mechanisms. This function of SPP might also be part of an ancient stress response since in *S. cerevisiae*, nutrient deprivation activates SPHK, and yeast mutants with decreased SPHK activity also displayed an increased sensitivity to heat stress (Lanterman and Saba, 1998, supra). Furthermore, accumulation of phosphorylated long chain sphingoid bases in *S. cerevisiae* due to the deletion of *LBP1* and *LBP2* genes, which encode specific SPP phosphatases, results in dramatically enhanced survival upon severe heat shock (Mandala et al., 1998, supra).

SPP has been shown to inhibit cell motility, chemoinvasion, and haptotactic motility (Sadahira et al., 1994, *FEBS Lett.* 340, 99-103) of human B16 melanoma cells and other cell lines in a low concentration range (10 to 100 nM) (Sadahira et al., 1992, supra), and these effects seem to be mediated through a cell surface receptor since SPP immobilized on glass beads, which can not traverse the cell membrane, mimicked the effects of SPP (Yamamura et

al., 1997, supra). SPP also inhibits chemotactic motility and trans-endothelial migration of human neutrophils (Kawa et al., 1997, *FEBS Lett.* 420, 196-200). Unexpectedly, and in contrast to numerous reports, we have found that increased SPP levels as a result of overexpression of SPHK in NIH 3T3 fibroblasts, as well as in HEK293 cells, drastically inhibited chemotactic motility and random motility, indicating a role for endogenous SPP in regulation of cell motility. This is consistent with the conclusion that SPP, formed in response to PDGF, may play a role in PDGF-regulated motility of human arterial smooth muscle cells (Bornfeldt et al., 1995, supra). Moreover, we have found that only micromolar concentrations of SPP, as well as sphingosine, which is readily taken up by cells and phosphorylated to SPP, inhibit cell motility and chemoinvasion of the human breast cancer cell line MDA-MB-231,which lack the SPP receptor Edg-1 and specific binding of SPP (Wang et al., 1998, supra). Thus, SPP may act intracellularly, and not by binding to a putative cell surface receptor, to inhibit motility and invasion of MDA-MB-231 cells (Wang et al., 1998, supra).

The mechanism by which SPP elicits this anti-motogenic effect is not yet known. Intracellular SPP has been shown to induce rapid reorganization of the actin cytoskeleton (Bornfeldt et al., 1995, supra; Wang et al., 1997, supra), resulting in stress fiber formation and concomitant assembly of focal adhesions as well as tyrosine phosphorylation of both focal adhesion kinase (FAK) and paxillin in Swiss 3T3 fibroblasts (Wang et al., 1997, supra). It is still not clear whether SPP acts extracellularly through an Edg family receptor to inhibit motility. Moreover,

more recently it has been shown that SPP, by binding to a cell surface receptor, stimulated, rather than inhibited, invasion of T-lymphoma cells into a fibroblast monolayer (Stam et al., 1998, *EMBO J.* 17, 4066-4074). Thus, the effect of SPP on cell motility may be cell type specific and depend on the expression of different SPP receptors and further studies are needed to clarify these issues.

In summary, this study substantiates a role for SPHK-derived SPP as a second messenger in cell proliferation, survival, and motility. SPHK belongs to a new class of lipid kinases, different in structure and biochemical properties than the PI kinase family, but similar in the broad spectrum of signals and in vital and versatile cell functions that they regulate.

What is claimed is:

1. An isolated sphingosine kinase DNA fragment or any portion thereof.

2. An isolated and purified DNA fragment which encodes a sphingosine kinase.

3. An isolated and purified DNA fragment which encodes a peptide of murine sphingosine kinase, said DNA fragment comprising the sequence specified in Genbank Accession no. AF068748, or GenBank Accession no. AF068749, or a polynucleotide fragment of said sequence comprising at least 30 nucleotides.

4. An isolated and purified sphingosine kinase DNA fragment according to claim 2 which encodes 381 amino acids of sphingosine kinase or a natural variant or synthetic variant thereof encoding sphingosine kinase, or a peptide fragment thereof comprising at least 10 amino acids.

5. A recombinant DNA construct comprising:
(i) a vector, and
(ii) the sphingosine kinase DNA fragment of claim 1.

6. A recombinant DNA construct comprising:
(i) a vector, and
(ii) the sphingosine kinase DNA fragment of claim 3.

7. A recombinant DNA construct according to claim 6, wherein said vector is an expression vector.

8. The recombinant DNA construct according to claim 6, wherein said vector is a prokaryotic vector.

9. The recombinant DNA construct according to claim 6, wherein said vector is a eukaryotic vector.

10. A host cell transformed with a recombinant DNA construct according to claim 6.

11. A host cell according to claim 10, wherein said cell is prokaryotic.

12. A host cell according to claim 10, wherein said cell is eukaryotic.

13. A method for producing sphingosine kinase peptide which comprises culturing the cells according to either claim 11 or 12, under conditions such that said DNA fragment is expressed and said sphingosine kinase peptide is thereby produced.

14. An isolated recombinant sphingosine kinase produced by the method of claim 13.

15. A method for screening agents or drugs which reduce or eliminate sphingosine kinase activity said method comprising detecting a decrease sphingosine kinase enzyme activity in the presence of said agent or drug.

16. A method for detecting sphingosine kinase in a sample comprising

(i) contacting a sample with antibodies which recognize sphingosine kinase; and

(ii) detecting the presence or absence of a complex formed between sphingosine kinase and antibodies specific therefor.

17. An antibody to a peptide having the amino acid sequence specified in SEQ ID NO:1, or any portion thereof.

18. A method for detecting agents or drugs which inhibit sphingosine kinase activity, said method comprising:

(i) delivering a recombinant DNA construct according to claim 5 into a cell such that sphingosine kinase is produced in said cell;

(ii) adding at least one drug or agent to said cell alone or in combination; and,

(iii) detecting whether or not said drug or agent inhibits sphingosine kinase activity by measuring sphingosine kinase-dependent phosphorylation of lipids in said cell and comparing it to a control which did not receive said drug or agent wherein a decrease in the amount of sphingosine kinase-dependent phosphorylation of lipids as compared to control indicates an inhibitory drug or agent.

19. A method for detecting agents or drugs which promote sphingosine kinase activity, said method comprising:

(i) delivering a recombinant DNA construct according to claim 5 into a cell such that sphingosine kinase is produced in said cell;

(ii) adding at least one drug or agent to said cell alone or in combination; and,

(iii) detecting whether or not said drug or agent stimulates sphingosine kinase activity by measuring

sphingosine kinase-dependent phosphorylation of lipids in said cell and comparing it to a control which did not receive said drug or agent wherein an increase in the amount of sphingosine kinase-dependent phosphorylation of lipids in said cell as compared to control indicates a stimulatory drug or agent.

20. An agent or drug capable of inhibiting sphingosine kinase activity.

21. An agent or drug capable of promoting sphingosine kinase activity.

22. A therapeutic compound comprising said agent or drug according to claim 20 for use in cancer treatment.

23. A method for detecting sphingosine kinase in a sample using the polymerase chain reaction.

24. A diagnostic kit for detecting sphingosine kinase RNA/cDNA in a sample comprising primers or oligonucleotides specific for sphingosine kinase RNA or cDNA suitable for hybridization to sphingosine kinase RNA or cDNA and/or amplification of sphingosine kinase sequences and suitable ancillary reagents.

25. A method for measuring sphingosine-1-phosphate in a sample comprising

(i) separating sphingosine-1-phosphate (SPP) from other phospholipids to produce isolated SPP;

(ii) converting isolated SPP to sphingosine producing converted sphingosine;

(iii) phosphorylating said converted sphingosine using a detectably labeled phosphate; and